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13. ABSTRACT (Maximum 200 Words)

In this proposal we have investigated the hypothesis that overexpression of a novel truncated version of AIB1 (Δ3AIB1) that we have found to be overexpressed in breast cancer is important for tumor development by impacting upon nuclear hormone receptor function. We have examined the hypothesis that the novel AIB1 variant has an altered function that changes its interaction with nuclear receptors such as the estrogen or progesterone receptor. We propose that changes in the level of expression of the novel AIB1 variant will support tumor progression and may well have prognostic significance for breast cancer. We have now determined that Δ3AIB1 is overexpressed relative to the full-length protein in breast cancer tumor samples and cell lines. We have also determined that Δ3AIB1 is a significantly more active coactivator that full-length AIB1 on the estrogen and progesterone receptor (*J. Biol. Chem.* 276, 39736-39741, 2001). Δ3AIB1 also plays a role in tamoxifen resistance and its overexpression makes tamoxifen a much more potent estrogen (*Oncogene* 23, 403-409, 2004). In addition, a surprising finding is that overexpression of Δ3AIB1 can also potentiate EGF signaling. This implies that Δ3AIB1 can also drive hormone and non-hormone mediated proliferation in breast cancer.

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Introduction

An area of chromosome 20q, frequently amplified in breast cancer, harbors a steroid receptor coactivator (SRC) gene, AIB1 (amplified in breast cancer 1). (1) SRCs are a family of proteins that bind to the liganded receptor and, when overexpressed, are able to increase the maximal response to steroids such as estrogen and progesterone. (2) The AIB1 gene is amplified in 5-10% of breast cancers and the mRNA is overexpressed in about 60% of tumors. (1) We have also determined that the AIB1 protein is overexpressed in breast cancer by immunocytochemical analysis. (3) The role of AIB1 is further complicated by overexpression studies that demonstrate that AIB1 can not only potentiate the action of the estrogen and progesterone receptor, but also a number of the nuclear receptors in transient transfection overexpression assays. (2) Interestingly, some of these receptors such as retinoid and vitamin D_3 have predominantly anti-proliferative and pro-apoptotic roles in breast cancer whereas other receptors such as estrogen, progesterone and thyroid receptor are considered to induce growth by increased proliferation and reduced apoptosis. Thus, it is possible to envisage the overexpression of AIB1 as enhancing both proliferative and anti-proliferative effects of nuclear receptors. It is also apparent that the effects of the SRC coactivators are not confined to interactions with the nuclear receptor family. The SRC proteins also interact with other co-factors such as the pCAF and CBP/p300 co-integrators (4) that are recipients of signals from a number of cellular signaling pathways. In addition, AIB1 has been described as interacting with p53. (5) Thus, it is entirely possible that AIB1 may have wide-ranging effects on cell cycle and proliferation that are independent of its effects on nuclear receptor function.

Another aspect of nuclear coactivators which is particularly interesting is the existence of exon splice variants of the SRC-1 and SRC-3 (AIB1) genes that can be predicted from the cDNA cloning data, although levels of expression and impact of the presence of these alternative splice products on coactivator function is not known. (2) For ACTR/AIB1, such putative splice variants can be predicted within the receptor interacting region and in the N-terminal helix-loop-helix-(HLH)-Per-Arnt-Sim (PAS) domain. We have now detected a novel Δ exon 3 AIB1 splice variant that is expressed in MCF-7 breast cancer cells as well as in breast tumor tissue. (Appendix manuscript) We have determined the exon intron structure of AIB1 from the human genome database and the predicted protein product from this isoform is an N-terminally truncated version of AIB1 that has a deleted HLH domain and most of the PAS region removed. The predicted size of this protein is approximately 125 kDA and a protein with this molecular weight is detected in our Western blot analysis of MCF-7 cells and is translated *in vitro* from the Δ exon 3 splice AIB1 cDNA.

To our knowledge this is the first report of expression of an AIB1 splice variant at the mRNA and protein levels in breast cancer cells. Alterations in the domain are of interest since it has been shown that the PAS/HLH domain can mediate homodimeration between family members and, in this case, may mediate crosstalk between nuclear receptors and PAS/HLH family members that have wide-ranging effects in cellular growth control.

In this grant we have examined the hypothesis that the Δ exon 3 splice AIB1 variant has an altered function, due to the removal of the PAS/HLH domain, which changes its interaction with nuclear receptors or other transcription regulators. We propose that changes in the level of expression of Δ exon 3 AIB1 will support tumor progression and will likely have prognostic significance for breast cancer.

Body

Statement of Work

Task 1. Analyze the function of the Δ exon 3 AIB1 isoform versus full-length AIB1 in transient transfection assays.

We first compare the Δ exon 3 AIB1 to the full-length AIB1 protein with respect to their ability to potentiate nuclear receptor function in transient transfection assays in transformed and non-transformed breast cell lines.

We have now completed a series of experiments showing the Δ exon 3 isoform is a significantly more effective transcriptional coactivator of both the estrogen receptor and the progesterone receptor. We recently had a paper accepted (*Oncogene*, appendix publication) showing that Δ3AIB1 potentiates partial agonists (tamoxifen) and natural estrogens (genistein). (See appendix manuscript) In addition, we have now shown that the Δ exon 3 AIB1 variant potentiates epidermal growth factor signaling. The data we have obtained on the functional impact of the expression of Δ exon 3 AIB1 in transient systems and the expression data showing that Δ exon 3 mRNA is highly overexpressed in both breast cancer cell lines and breast tumor samples has now been published in a *J. Biol. Chem.* paper and this manuscript can be found in the appendix of this report.

Task 2: Analyze the effect of stable expression of Δ exon 3 AIB1.

We will examine the impact of the expression of the Δ exon 3 AIB1 on normal breast epithelial and on breast cancer cell phenotype by developing cell lines stably expressing the Δ exon 3 AIB1 or full-length AIB1. We will focus in particular on malignant transformation in the normal epithelial cells, and on basal and hormone-induced proliferation and apoptosis in the cancer cells.

This task is currently in progress. We are trying to create cell lines that stably express the Δ exon 3 and to help us with that, we have now made a tagged version of the Δ exon 3 expression vector both with a histidine tag at the C-terminus and also a MYC tag at the C-terminus to determine if we have cell lines that clearly overexpress Δ exon 3. We have tried to make cell lines overexpressing Δ3AIB1 and these cells do not survive. We succeeded in making Δ3AIB1 Tet-inducible stable cell lines and now will examine changes in these levels on phenotype and tumorigenicity, but unfortunately the fusion protein that was made was truncated. To overcome this, we have recently started to work with adenovirus infectious primary epithelial cell lines from mice. Hopefully this approach will overcome some of the problems we have had with these cell lines.

Task 3: Determine the effect of overexpression of Δ exon 3 AIB1 on angiogenic and invasive properties of breast cancer cells.

We will use cell lines or adenovirus infected primary cells developed under *Task 2* to determine the effects of Δ exon 3 AIB1 on stromal and endothelial cell interactions with breast tumor cells.

We have some preliminary data showing that reducing AIB1 in cells does impact the invasive behavior of tumors, and we will now extend this with the Tet-inducible cell lines, or the adenovirus infected primary mammary epithelial lines. For this, we are encouraged that this Δ exon 3 AIB1 splice variant will have a major impact n the angiogenic and invasive phenotype on MCF-7 cells and will continue to persevere with this task in the future, using the cell lines we are currently developing under *Task 2*.

Key Research Accomplishments

Task 1: Completed in MCF-7 cells. *J. Biol. Chem.* Article attached. *Oncogene* Manuscript attached.
Task 2: Our new approach of adenovirus infection of primary mammary epithelium is underway.
Task 3: In progress; as soon as adenovirus infection of mammary epithelial cell lines occurs.

Reportable Outcomes
Article
Reiter, R., Oh, A.S., Wellstein, A. and **Riegel, A.T**. "Impact of the Nuclear Receptor Coactivator AIB1 Isoform AIB1- Δ3 on Estrogenic Ligands with Different Intrinsic Activity." *Oncogene* (2004) 23, 403-409.

Reiter, R., Wellstein, A., and **Riegel, A.T.** "An Isoform of the Coactivator AIB1 that Increases Hormone and Growth Factor Sensitivity is Overexpressed in Breast Cancer." *J. Biol. Chem.* (2001) 276, 39736-39741.

Abstracts

"An isoform of AIB1 (Δ3AIB1) increases estrogenic activity of both agonists and partial agonists of the estrogen receptor." Ronald Reiter, Annabell Oh, Kristina J. Lauritsen, Anton Wellstein and **Anna T. Riegel** – AACR Annual Meeting, Washington, DC July 2003

"The Nuclear Receptor Coactivator AIB1 has a rate limiting role in growth factor dependent growth in MCF-7 breast cancer cells." Annabell Oh, Heinz-Joachim List, Ronald Reiter, Anton Wellstein and **Anna T. Riegel** – AACR Annual Meeting, Washington, DC July 2003

Reiter, R., Wellstein, A. and **Riegel, A.T.** "An Isoform of the Coactivator AIB1 that Increases Hormone and Growth Factor Sensitivity is Overexpressed in Breast Cancer. " Poster presentation for the Era of Hope Meeting, Orlando, FL, September 2002.

Patent

"Coactivators in the Diagnosis and Treatment of Breast Cancer." #RIAN432801 – Granted.

Conclusions

In this study we have provided evidence of a splice variant of AIB1 that has exon 3 deleted. The AIB1-Δ3 mRNA is translated *in vivo* in breast cancer cells into an NH2-terminal truncated form of AIB1 that has several properties of interest. The first is that, on a per molecule basis, it is a more potent transcriptional coactivator of both the estrogen and progesterone receptors that the full-length AIB1 protein. In addition, overexpression of AIB1-Δ3 can increase the estrogenic activity of tamoxifen and natural estrogens. This may explain why AIB1 overexpression can be related to tamoxifen resistance in some tumors. This result was unexpected given that previous studies of NH2-terminal deletion mutants of the AIB1-related protein SRC-1 did not reveal an impact on nuclear receptor signaling.

The second interesting aspect of the function of the AIB1-Δ3 isoform was that it also potently increased EGF signaling in ME-180 squamous carcinoma cells.

In addition, of major interest for breast cancer is our finding that the AIB1-Δ3 mRNA is overexpressed in breast cancer cell lines and in human breast tumors. Our analysis of tumor cell lines suggest that there is an overall increase in the AIB1-Δ3 mRNA relative to the full-length AIB1, although we do not know whether this is related to the gene amplification status of the endogenous gene.

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The Nuclear Receptor Coactivator AIB1 Mediates Insulin-like Growth Factor I-induced Phenotypic Changes in Human Breast Cancer Cells

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ABSTRACT

The nuclear receptor coactivator AIB1 (amplified in breast cancer 1) is overexpressed in human breast cancers and is required for estrogen signaling. However, the role of AIB1 in breast cancer etiology is not known. Here, we show that AIB1 is rate-limiting for insulin-like growth factor I (IGF-I)-dependent phenotypic changes and gene expression in human breast cancer cells. Reduction of endogenous AIB1 levels by small interfering RNA in MCF-7 breast cancer cells prevented IGF-I–stimulated anchorage-independent growth by reducing IGF-I–dependent anti-anoikis. cDNA array and immunoblot analysis of gene expression revealed that reduction in AIB1 levels led to a significant decrease in the expression of several genes controlling the cell cycle and apoptosis. These AIB1-dependent changes were also observed in the presence of estrogen antagonist and were corroborated in the estrogen receptor-negative cell line MDA MB-231. AIB1 reduction decreased the expression of the IGF-I receptor and IRS-1 in MCF-7 but not in MDA MB-231 cells. IGF-I–stimulated activation of AKT was reduced by AIB1 small interfering RNA treatment, whereas mitogen-activated protein kinase (extracellular signal-regulated kinase 1/2) activation by IGF-I was unaffected. We conclude that AIB1 is required for IGF-I–induced proliferation, signaling, cell survival, and gene expression in human breast cancer cells, independent of its role in estrogen receptor signaling.

INTRODUCTION

The nuclear receptor coactivator AIB1 belongs to the p160/SRC (steroid receptor coactivator) family consisting of SRC-1 (1), TIF-2 (GRIP1; ref. 2) and AIB1 (ref. 3; ACTR/RAC3/TRAM-1/SRC-3; refs. 4–7). The AIB1 gene is amplified in several human cancers, such as breast, ovarian, pancreatic, and gastric cancer (3, 8, 9). Amplification of the AIB1 gene is detected in 5 to 10% of primary breast tumors and AIB1 is highly expressed in many breast tumor specimens (3, 10–12). AIB1 enhances *in vitro* the transcriptional activity of the estrogen receptor (ER; refs. 3, 4, 7) and binds directly to ER *in vivo* (13). Furthermore, AIB1 is rate-limiting for estrogen-mediated growth of MCF-7 human breast cancer cells (14). AIB1 gene expression is up-regulated by selective ER modulators, such as tamoxifen (15) and the estrogenic activity of selective ER modulators, can be increased by AIB1 and an AIB1 isoform (16). However, emerging data suggest that the role of AIB1 is not restricted to nuclear receptor signaling. Disruption of p/CIP, the mouse homologue of AIB1, results in a pleiotropic phenotype, including reduced female reproductive function and blunted mammary gland development in mice (17, 18). Interestingly, embryonic tissues from p/CIP-knockout mice show severe defects in the insulin-like growth factor I (IGF-I) and growth hormone-signaling pathways (18). Consistent with this role for AIB1 in growth factor signaling, we have found that an isoform of AIB1 (Δ3-AIB1) overexpressed in breast tumors strongly enhances epidermal growth factor-mediated transcription in squamous cell carcinoma cells (19). Also, AIB1 overexpression is positively correlated with the expression of p53 and HER2/neu in breast tumors (20). p/CIP also plays a role in CREB binding protein–dependent transcriptional activation induced by IFN-γ and 12-*O*-tetradecanoylphorbol-13-acetate (21), and a study of Taiman, the Drosophila homologue of AIB1, indicates that AIB1 is also involved in the control of cell motility as well as platelet-derived growth factor/vascular endothelial growth factor signaling (22, 23). Taken together, these data suggest that AIB1 may well be an important factor for growth factor-mediated signaling pathways. In this study, we report that selective reduction of endogenous AIB1 levels in MCF-7 cells reveals a significant role for this coactivator in IGF-I signaling in human breast cancer cells.

MATERIALS AND METHODS

Cell Culture and Reagents. MCF-7 and MDA MB-231 human breast cancer cells were maintained in Improved Modified Eagle's Medium (IMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum. Recombinant human IGF-I (R&D Systems, Minneapolis, MN) was resuspended in 10 mmol/L acetic acid +0.1% bovine serum albumin and used at 100 ng/mL (13 nmol/L). ICI 182,720 (Tocris Cookson, Ellisville, MO) was resuspended in etomidate and used at a concentration of 10 nmol/L.

Small Interfering (si)RNA Design. Twenty-one–mer oligoribonucleotides of sense and antisense RNA strands were synthesized corresponding to nucleotides 564–582 of the AIB1 coding region. The AIB1 sense oligoribonucleotide sequence as follows: r(GGUGAAUCGAGACGGAAAC)dTT. The AIB1 antisense oligoribonucleotide sequence as follows: r(GUUUCCGUCUCGAUUCACC)dTT. The control siRNA is a scrambled sequence and does not target any known mammalian mRNA (Qiagen, Valencia, CA). The control sense oligoribonucleotide sequence as follows: r(UCCGUUUCGGUCCACAUUC)dTT. The control antisense oligoribonucleotide sequence as follows: r(GAAUGUGGACCGAAACGGA)dTT (Qiagen). The lyophilized double-stranded RNA was reconstituted in 1 mL of annealing buffer [100 mmol/L potassium acetate, 30 mmol/L HEPES-KOH, 2 mmol/L magnesium acetate (pH 7.4)], giving a final concentration of 20 μmol/L. The solution was then heated for 1 minute at 90°C and incubated at 37°C for 60 minutes.

Attached Cell Proliferation, Cell Cycle, and Apoptosis Assays. For siRNA experiments, 24 hours before transfection, MCF-7 cells were plated in a 10-cm dish at 50% confluency in IMEM +10% FBS. For each transfection, 60 μL of 20 μmol/L AIB1 siRNA were diluted with 1 mL of IMEM plus 60 μL of Oligofectamine (Invitrogen). The siRNA and Oligofectamine solutions were allowed to complex at room temperature for 15 minutes before treatment of the cells. After washing, the siRNA-Oligofectamine complex was added to the cells in 5 mL of IMEM and incubated for 4 hours at 37°C. A total of 1.5 mL of IMEM +30% FBS was added to the transfected cells and incubated for 16 to 18 hours at 37°C. For proliferation assays, the transfected cells were trypsinized and placed into 96-well plates and treated with IMEM +1% charcoal-stripped calf serum (CCS) containing 100 ng/mL IGF-I or 10% FBS (serum). Cell number was determined by a WST-1 colorimetric assay (Roche Diagnostics, Indianapolis, MN). For cell cycle or apoptosis analysis, the transfected cells were trypsinized and placed into 60-mm dishes and treated with IMEM +1% CCS with 100 ng/mL IGF-I or 10% FBS for 24 hours. Cell cycle analysis was done with the Vindelov method of nuclei preparation for flow cytometry DNA analysis. The percentage of cells in early and late

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apoptosis (percent cell death) was analyzed by staining with Annexin V-FITC (Trevigen, Inc., Gaithersburg, MD).

Soft Agar Colony Formation Assay. MCF-7 cells were transfected with either AIB1 or control siRNA as described from the siRNA proliferation assays. After 16 to 18 hours, 7000 cells were resuspended in 0.35% soft agar and layered on top of 1 mL of 0.6% solidified agar in a 35-mm dish with 100 ng/mL IGF-I or 10% FBS (serum). IMEM +1% CCS were included in both layers. The soft agar colonies were allowed to grow at 37°C for 10 to 15 days. Cell colonies with a diameter of $\geq$80 μm were counted with an image analyzer (Omnicon TCA, Biologics, Gainesville, VA). Experiments were carried out in triplicate.

Cell Suspension Cell Cycle and Apoptosis Assays. To prevent cell attachment, 60-mm dishes were coated with 10 mg/mL poly(2-hydroxyethyl methacrylate; poly-HEMA, Sigma-Aldrich, St. Louis, MO) diluted in etomidate and allowed to dry completely. Estrogen-stripped MCF-7 cells were transfected with AIB1 or control siRNA as described above for the proliferation assays. After 48 hours, the transfected cells were plated onto the poly-HEMA coated dishes in IMEM +1% CCS with 100 ng/mL IGF-I or 10% FBS (serum). After 24 hours, the cells were harvested either for cell cycle analysis or apoptosis analysis as described above. Each experiment was carried out in duplicate.

Western Blot Analysis. Attached cells were transfected with siRNA and treated with growth factors for 48 hours. Unattached cells (poly-HEMA), a pool of MCF-7 or MDA MB-231 cells, was transfected with siRNA under normal adherent culture conditions. After 24 hours, the transfected cells were trypsinized, washed in 5% CCS + IMEM, and plated in poly-HEMA coated dishes for 24 hours with appropriate treatment media. Cells were then washed with cold PBS, lysed in 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 40 mmol/L β-glycerophosphate-Na, 0.25% Na-deoxycholate, 1% NP40, 50 mmol/L NaF, 20 mmol/L Na PP_I, 1 mmol/L EGTA, 1 mmol/L Na_3VO_4, and 1× complete protease inhibitor (Roche Diagnostics) and incubated on ice for 20 minutes. The lysate was centrifuged at 10,000 × g at 4°C for 15 minutes. The lysate was boiled in SDS-PAGE buffer with reducing agents, proteins were resolved by electrophoresis on a 4 to 20% Tris-glycine gel, transferred to a polyvinylidene difluoride membrane, and the membrane was incubated for 1 hour at room temperature with 5% milk in PBST (PBS, 0.2% Tween 20). Primary and secondary antibodies were diluted in 5% milk in PBST, and incubations were done at room temperature for 1 hour. The antibodies used were raised against: AIB1 (BD Transduction Laboratories, San Jose, CA); β-actin (Chemicon International, Inc., Temecula, CA); IRS-1 (Upstate, Lake Placid, NY); IRS-2 (Upstate); ER-α clone 1D5 (DakoCytomation, Carpinteria, CA); cyclin D1 (Neomarkers/Lab Vision, Fremont, CA); Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA); and IGF-I receptor (IGF-IR) α, HER2/erbB2, phospho-AKT (Ser^{473}), AKT, phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}), and p44/42 MAPK (Cell Signaling Technologies, Beverly, MA). Relative band intensities were assessed with densitometry and corrected for β-actin loading.

Real-Time Reverse Transcription-PCR. Total RNA was extracted and DNase treated with the RNeasy Mini kit (Qiagen). Real-time reverse transcription-PCR was done with the SuperScript One-Step Reverse Transcription-PCR with Platinum Taq system (Invitrogen). Samples were reverse transcribed for 30 minutes at 58°C, followed by a denaturing step at 95°C for 5 minutes and 40 cycles of 15 s at 95°C and 1 minute at 58°C. Fluorescence data were collected during the 58°C step with the Cycler iQ Detection System (Bio-Rad Laboratories, Hercules, CA). The primers and probes for real-time reverse transcription-PCR measurement were as follows: AIB1 forward primer, 5′-CAGTGATTCACGAAAACGCA-3′; AIB1 reverse primer, 5′-CAGCTCAGCCAATTCTTCAAT-3′; AIB1 probe, 6FAM-TGCCATGTGATACTCCAG AAG-Black Hole Quencher 1 (BHQ1); glyceraldehyde-3-phosphate dehydrogenase forward primer, 5′-CCCACATGGCCTCCAAGGAGTA-3′; glyceraldehyde-3-phosphate dehydrogenase reverse primer, 5′-GTGTACATGGCAACTGTGAGGAGG-3′; and glyceraldehyde-3-phosphate dehydrogenase probe, 6FAM-ACCCCTGGACCAGCCCCAGC-TAMRA.

cDNA Array Analysis. The method used to prepare samples for cDNA microarray analysis is outlined in the Affymetrix Gene Chip Expression Analysis Technical Manual, Section 2: Eukaryotic Sample and Array Processing. Total RNA was harvested from estrogen-stripped MCF-7 cells transfected with AIB1 or control siRNA for 48 hours (RNeasy Mini kit, Qiagen). Twenty micrograms of total RNA were used to synthesize double-stranded cDNA. T7 oligo(dT) primers [5′-GGCCAGTGAATTGTAATACGACTCACTATAGG-GAGGCGG-$(dT)_{24}$-3′] were used to prime the first-strand cDNA synthesis. Synthesis of biotin-labeled cRNA from double-stranded cDNA was done with the Enzo BioArray High Yield RNA Transcript Labeling kit (Enzo Life Sciences, Farmingdale, NY). Twenty micrograms of fragmented biotin-labeled cRNA were hybridized to the Affymetrix Human Genome U133A GeneChip (Affymetrix, Santa Clara, CA). MCF-7 cells either transfected with the control or AIB1 siRNA were analyzed using four U133A chips.

Array Analysis. For each of the eight arrays, measurements from 22,283 genes were obtained. For each gene, an indicator of its expression level is given as either present, absent, or marginal call because it is assigned by Affymetrix Microarray suite software. Gene expression measurements that were <10 were given a threshold value of 10. Log base 2 transformations were applied to the gene expression values to reduce variation and to make the data

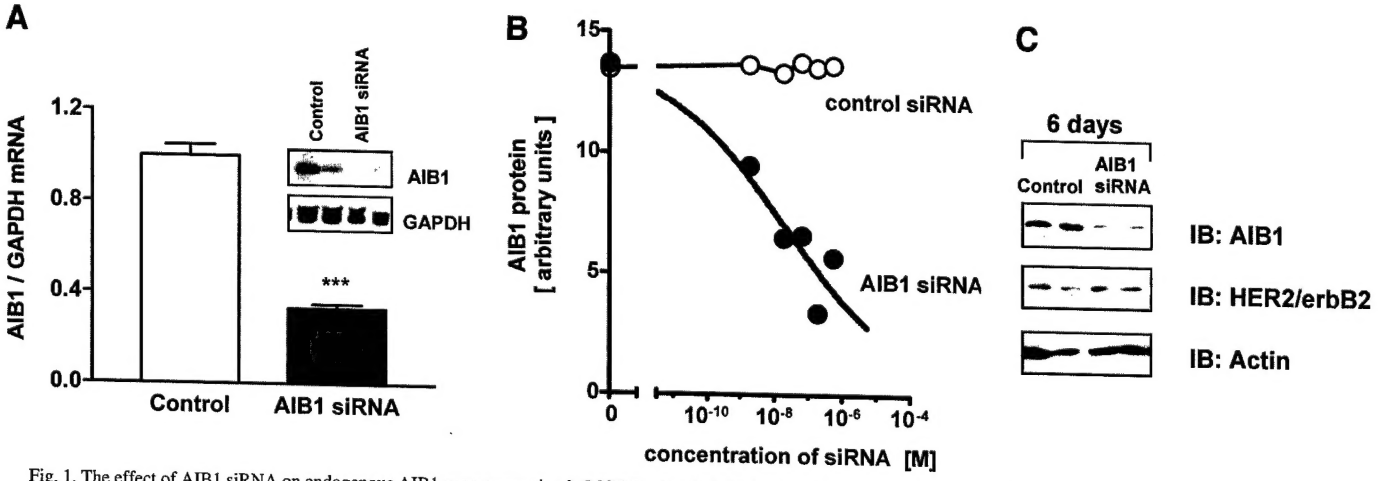

Fig. 1. The effect of AIB1 siRNA on endogenous AIB1 gene expression in MCF-7 cells. *A*. A 21-bp double-stranded RNA was designed against exon 7 of AIB1 mRNA. The control siRNA is also a 21-bp dsRNA but does not have any known homology to eukaryotic genes. The levels of AIB1 message after AIB1 siRNA transfection were examined by real time PCR and Northern blotting (*inset*). For real-time PCR analysis, total RNA was harvested from MCF-7 cells 48 hours after they were transfected with 2 × 10^{-7} mol/L AIB1 siRNA or control siRNA. Samples were run in duplicate and are normalized relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Cytoplasmic RNA was used for Northern blot analysis. To detect AIB1 mRNA, a 7.5-kb *Eco*RI fragment from pCMX-ACTR A38 (703–927 aa) was used as a probe. Quantitation of the Northern blotting was done with a phosphoimager and normalized relative to GAPDH. The graph represents the mean ± SE of four independent experiments. ***$P < 0.01$, Student's t test. *B*, concentration response of siRNA on AIB1 protein levels. Total cell lysates were harvested 48 hours after transfection and analyzed by Western blotting. AIB1 protein levels were quantitated by densitometry and normalized relative to β-actin. Nonlinear logistic regression analysis was used to determine the IC_{50}, 2 × 10^{-8} mol/L (± 7.8% SE). On the basis of this, 2 × 10^{-7} mol/L was the concentration of siRNA used in all subsequent experiments. *C*. AIB1 siRNA effectively knocks down protein expression up to 6 days after transfection of 2 × 10^{-7} mol/L AIB1 siRNA. No changes in the expression of both the HER2/erbB2 receptor and β-actin show that the siRNA specifically targets AIB1 protein and does not have nonspecific effects. Total cell lysates of were harvested 6 days after the transfection.

more normally distributed. Each gene that had an absent call from five or more of eight measurements was eliminated. This reduced the data set to 12,057 genes. The following analysis was done on the 12,057 genes with BRB-Array Tools (V.3.0.1). The intensity values were first normalized such that the median log intensity of each of the eight arrays is equal to the median log intensity for the reference array (BRB array tools arbitrarily choose the second array to be the reference array). Two sample *t* tests were carried out to compare the expression intensity between AIB1 high (control siRNA transfected) and AIB1 low (AIB1 siRNA transfected) arrays for each of the 12,057 genes with four arrays per group using the randomized variance model. A total of 124 genes showed a statistically significant difference between AIB1 high and AIB1 low at $P \leq 0.0025$ from the univariate test. The 124 genes include genes that were up or down-regulated by AIB1. The genes were also ranked according to *P* value.

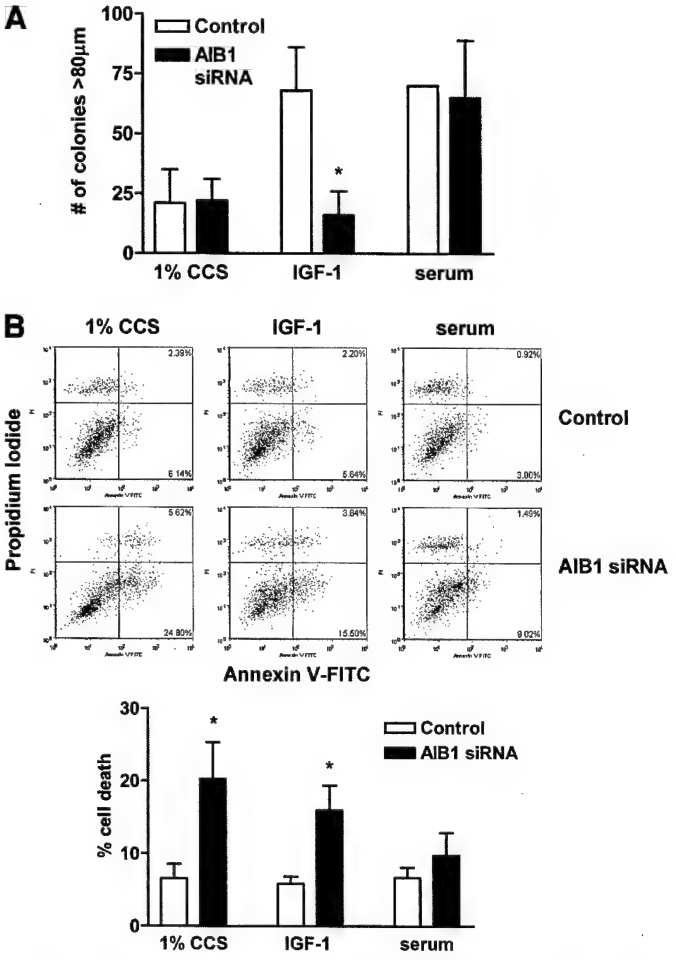

Fig. 2. AIB1 is critical for the growth and survival of MCF-7 cells under anchorage-independent conditions. *A*, soft agar colony formation of MCF-7 cells. A pool of MCF-7 cells was transfected with either AIB1 siRNA or control siRNA for 24 hours. The transfected cells were trypsinized, washed with IMEM +1% CCS, and plated in 0.35% soft agar dishes in the absence (1% CCS) or presence of 100 ng/mL IGF-I or 10% fetal bovine serum (serum). The colonies were measured after ~2 weeks. Colonies with a diameter ≥80 μm were counted with the Omnicon image analyzer. Experiments were carried out in triplicate. This graph is a representative figure out of three independent experiments. *Error bars* indicate SD. *, $P < 0.01$, relative to control siRNA sample. Student's *t* test. *B*, analysis of anoikis after AIB1 siRNA transfection. The cells were transfected with siRNAs as in *A*. After transfection, the cells were plated onto poly-HEMA–coated 60-mm dishes, a hydrogel that prevents cell attachment. The cells were treated with either 1% CCS, IGF-I or serum for 24 hours and harvested for apoptosis analysis by staining the cells with FITC-conjugated Annexin V and propidium iodide and performing a fluorescence-activated cell sorting analysis (Trevigen, Inc.). *Top panel:* cell death represents the total percentage of cells in early apoptosis (*bottom right quadrant* of the fluorescence-activated cell sorting analysis) and late apoptosis (*top right quadrant* of the FACS analysis). *Bottom panel:* the graph represents the mean ± SE of three independent experiments. *, $P < 0.01$, relative to control siRNA sample. Student's *t* test.

Table 1 *AIB1 siRNA effects on cell cycle progression of MCF-7 cells*

A. Cell cycle status of cells in suspension

	% (G_2 + S) phase	
Treatment	Control siRNA	AIB1 siRNA
1% CCS	25.5 (2.3)	23.1 (2.3)
IGF-I	33.2 (2.8)	31.9 (2.0)
Serum	32.2 (2.5)	28.5 (2.6)

B. Cell cycle status of attached cells

	% (G_2 + S) phase	
Treatment	Control siRNA	AIB1 siRNA
1% CCS	27.1 (0.6)	17.9 (4.4)
IGF-I	39.4 (3.6)	29.7 (5.8)
Serum	46.7 (4.4)*	41.6 (7.4)*

NOTE. Cell cycle analysis of suspended cells (A) and attached cells (B). MCF-7 cells were transfected with siRNA for 24 hours. Transfected cells were replated in poly-HEMA–coated (A) or –uncoated (B) 60-mm dishes and treated with 1% CCS and IGF-I or serum for 24 hours. Cells were then harvested and stained with the Vindelov method to determine the percentage of cells in each phase of the cell cycle. The percentage represents the mean (SD) of two independent experiments done in duplicate. An ANOVA analysis was done for control and AIB1 siRNA conditions and for suspension growth and attached cells. A significant difference (*, $P < 0.05$) was only found for serum stimulation relative to 1% CCS.

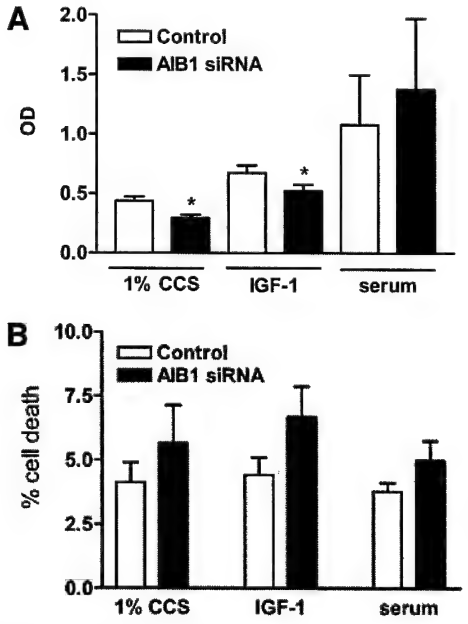

Fig. 3. Reduction of AIB1 decreases the rate of anchorage-dependent proliferation without significant changes in the levels of cell death. *A*, reduction in basal rate of proliferation: MCF-7 cells were transiently transfected with AIB1 siRNA or control siRNA for 24 hours. A total of 4500 cells was placed into each well of a 96-well plate and treated with 1% CCS, IGF-I, or serum. Cell growth was measured by the WST-1 colorimetric assay up to 8 days. Each experiment was done in triplicate. The graphs represent the mean ± SE of three independent experiments. *, $P < 0.05$, Student's *t* test. *B*, cell death in attached conditions: 24 hours after siRNA transfection, cells were replated in 60-mm dishes and treated with 1% CCS, IGF-I, and serum. After 24 hours, the cells were harvested and examined for percentage of cells in early and late apoptosis by Annexin V staining (Trevigen, Inc.). The graph represents the mean ± SE of three independent experiments. There is no significant difference between the results at $P < 0.05$. Student's *t* test.

RESULTS

Effect of siRNA on AIB1 Gene Expression. To study the role of AIB1 in IGF-I signaling, we used siRNA directed at nucleotides 564–582 of AIB1 to selectively reduce AIB1 gene expression in the MCF-7 breast cancer cell line. This region bears no significant homology to other coactivators or sequences in the human genome database. The AIB1 siRNA reduced the AIB1 mRNA (Fig. 1*A*) and AIB1 protein in a concentration-dependent fashion (Fig. 1*B*). The

Table 2 *Entire list of altered genes from the cDNA array analysis of AIB1 high (control) versus AIB1 low (AIB1 siRNA)*

	Probe set	Mean expression for AIB1 high	Mean expression for AIB1 low	Fold (AIB high/AIB low)	Up (+) or down (−) regulated by AIB1	Rank of *P* of the randomized variance test	*P* of the randomized variance test
RAB, member of RAS oncogene family-like 4	205037 at	274.876	654.179	0.42	−	1	0.00002
Malic enzyme 1, NADP(+)-dependent, cytosolic	204058 at	440.668	1044.197	0.422	−	2	0.0000242
p53-induced protein PIGPC-1	217744 s at	571.677	1195.691	0.478	−	3	0.0000321
AIB1	209062 x at	1682.874	726.757	2.316	+	4	0.0000377
Cyclin D1	208712 at	3262.634	1292.907	2.523	+	5	0.0000426
Hypothetical protein FLJ10842	222132 s at	666.67	297.556	2.24	+	6	0.0000501
RAB27B, member RAS oncogene family	207017 at	44.51	111.832	0.398	−	7	0.0000511
Sialyltransferase 8D (α-2, 8-polysialytransferase)	206925 at	109.627	318.894	0.344	−	8	0.0000721
Chromagranin A (parathyroid secretory protein 1)	204697 s at	598.087	242.007	2.471	+	9	0.0000778
Chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	203666 at	551.907	258.868	2.132	+	10	0.000082
Histone 1, H2bd	222067 x at	1273.525	4863.035	0.262	−	11	0.0000901
AIB1	209060 x at	2886.879	1409.345	2.048	+	12	0.0000974
3′-Phosphoadenosine 5′-phosphosulfate synthase 2	203058 s at	81.223	179.569	0.452	−	13	0.0001073
	203231 s at	78.312	276.067	0.284	−	14	0.000118
Pirin	207469 s at	198.894	669.774	0.297	−	15	0.0001229
Hypothetical protein FLJ10842	211352 s at	2164.567	1043.125	2.075	+	16	0.0001234
PHD finger protein 10	221786 at	1404.191	697.794	2.012	+	17	0.0001291
Bone morphogenetic protein 7 (osteogenic protein 1)	211259 s at	307.814	140.98	2.183	+	18	0.0001298
Hypothetical protein FLJ10842	218568 at	399.582	190.925	2.093	+	19	0.0001303
MAPK-1	212271 at	1279.667	569.648	2.246	+	20	0.0001316
	203232 s at	132.079	312.431	0.423	−	21	0.0001405
Chromosome 6 open reading frame 56	204048 s at	541.053	1017.398	0.532	−	22	0.0001614
3′-Phosphoadenosine 5′-phosphosulfate synthase 2	203060 s at	125.006	297.243	0.421	−	23	0.0001616
Hypothetical protein MGC2963	221255 s at	836.521	1710.86	0.489	−	24	0.000166
PDZ-1 domain-containing 1	205380 at	699.117	211.344	3.308	+	25	0.0001706
PHD finger protein 10	221787 at	936.315	458.354	2.043	+	26	0.0001719
Karyopherin α 1 (importin α 5)	202056 at	424.574	181.884	2.334	+	27	0.0001824
Karyopherin α 1 (importin α 5)	202055 at	959.842	391.313	2.453	+	28	0.0001887
MHC class I polypeptide-related sequence B	206247 at	667.084	308.834	2.16	+	29	0.0001928
Sapiens cDNA FLJ20338 fis, clone HEP 12179	214079 at	1694.251	329.078	5.148	+	30	0.0001957
Molybdenum cofactor synthesis 2	218212 s at	1431.594	624.03	2.294	+	31	0.0002208
HMBA-inducible	202814 s at	528.328	934.206	0.566	−	32	0.0002883
Filamin B	208613 s at	823.305	389.677	2.113	+	33	0.0002931
Pleckstrin homology domain containing, family B (evectins) member 2	201411 s at	517.485	1117.563	0.463	−	34	0.000316
Kynureninase (L-lynurenine hydrolase)	217388 s at	277.552	626.48	0.443	−	35	0.0003291
Potassium channel, subfamily K, member 5	219615 s at	566.977	259.781	2.183	+	36	0.0003647
Methionyl aminopeptidase 2	202015 x at	27.966	59.198	0.472	−	37	0.0003691
Glyoxalase I	200681 at	5937.301	2780.38	2.135	+	38	0.0003918
S100 calcium binding protein A14	218677 at	1140.565	2211.092	0.516	−	39	0.0003937
Olfactomedin 1	213131 at	1061.422	570.195	1.862	+	40	0.0004057
Polymerase (RNA) II (DNA-directed) polypeptide D	214144 at	146.593	254.863	0.575	−	41	0.0004117
Butyrophilin, subfamily 3, member A3	38241 at	67.413	177.872	0.379	−	42	0.0004601
WW domain binding protein 11	217821 s at	293.572	492.855	0.596	−	43	0.0004607
Hydroxy prostaglandin dehydrogenase 15-(NAD)	211548 s at	244.116	100.061	2.44	+	44	0.0004619
Synaptotagmin I	203998 s at	133.708	249.236	0.536	−	45	0.0004762
Chromosome 6 open reading frame 56	204049 s at	588.212	1310.993	0.449	−	46	0.0005077
DICER-1	213229 at	2307.863	1114.54	2.071	+	47	0.000515
Dodecenoyl-Coenzyme A δ isomerase (3,2 trans-enoyl-Coenzyme A isomerase)	209759 s at	653.268	1380.144	0.473	−	48	0.0005196
RAB 15, member RAS oncogene family	59697 at	1070.718	574.105	1.865	+	49	0.0005535
Solute carrier family 25 (mitochondrial carrier; ornithine transporter) member 15	218653 at	823.559	404.301	2.037	+	50	0.0005662
Ectonucleoside triphosphoate diphosphohydrolase 1	207691 x at	145.162	271.393	0.535	−	51	0.0005858
DICER-1	212888 at	1579.524	687.527	2.297	+	52	0.00062
Zinc finger, DHHC domain containing 3	218078 s at	515.903	307.607	1.677	+	53	0.0006223
Cdc42 guanine nucleotide exchange factor (GEF) 9	203264 s at	41.278	101.05	0.408	−	54	0.0006257
Trophoblast-derived noncoading RNA	214657 s at	378.673	934.216	0.405	−	55	0.0006377
KIAA0657 protein	212776 s at	1433.679	769.539	1.863	+	56	0.0007141
Phospholipase A2, group XII	221027 s at	258.746	549.612	0.471	−	57	0.0007233
Solute carrier family 7 (cationic amino acid transporter, y_system), member 5	201195 s at	6410.213	2766.12	2.317	+	58	0.0007245
Sialyltransferase	204542 at	230.423	442.079	0.521	−	59	0.0007661
Lin-7 homology A (*C. elegans*)	206440 at	232.047	447.247	0.519	−	60	0.000769
Family with sequence similarity 16, member A, X linked	203974 at	933.932	388.651	2.403	+	61	0.0007798
	220033 at	123.138	200.674	0.614	−	62	0.0007865
	217403 s at	70.891	135.505	0.523	−	63	0.0008073
Golgi autoantigen, golgin subfamily a, 2	204384 at	250.411	536.387	0.467	−	64	0.0008243
Abhydrolase domain-containing 3	213017 at	300.266	612.578	0.49	−	65	0.0008298
Palmitoyl-protein thioesterase 1 (ceroid-lipofuscinosis, neuronal 1, infantile)	200975 at	2632.758	5182.401	0.508	−	66	0.0008407
Keratin 8	209008 x at	19559.105	10904.508	1.794	+	67	0.0008456
Sorting nexin 5	217792 at	1105.939	615.404	1.797	+	68	0.0008456
Translocase of outer mitochondrial membrane 22 homologue (yeast)	217960 s at	315.36	675.567	0.467	−	69	0.0008724
ATP-binding cassette, sub-family C (CFTR/MRP), member 3	208161 s at	253.505	697.4	0.364	−	70	0.0009091
Golgi phosphoprotein 2	217771 at	334.384	162.16	2.062	+	71	0.0009642

Table 2 *Continued*

	Probe set	Mean expression for AIB1 high	Mean expression for AIB1 low	Fold (AIB high/AIB low)	Up (+) or down (−) regulated by AIB1	Rank of *P* of the randomized variance test	*P* of the randomized variance test
Deiodinase, iodothyronine, type I	206457 s at	72.876	139.655	0.522	−	72	0.0009778
Solute carrier family 9 (sodium/hydrogen exchange), isoform 3 regulatory factor 1	201349 at	5586.867	3087.018	1.81	+	73	0.0009937
Sarcoma antigen	220793 at	180.675	351.721	0.514	−	74	0.0010088
Toll-like receptor 3	206271 at	103.563	186.645	0.555	−	75	0.0010269
Karyopherin α 1 (importin α 5)	202058 s at	434.526	260.88	1.666	+	76	0.0010678
Inhibin, β B (activin AB neta polypeptide)	205258 at	1782.915	1014.699	1.757	+	77	0.0011023
Damage-specific DNA binding protein 2, 48 kDA	203409 at	317.875	547.097	0.581	−	78	0.0011139
Spastic ataxia of Charlevoix-Saguenay (sacsin)	213262 at	46.648	23.442	1.99	+	79	0.0011206
Bone morphogenetic protein 7 (osteogenic protein 1)	209590 at	1062.787	584.447	1.818	+	80	0.0011361
Myristoylated alanine-rich protein kinase C substrate	201669 s at	656.271	1391.009	0.472	−	81	0.001137
Histone deacetylase 1	201209 at	1258.795	2076.041	0.606	−	82	0.00117
MHC class I region ORF	206082 at	178.538	377.635	0.473	−	83	0.0011727
Phosphodiesterase 4A, cAMP-specific (phosphodiesterase E2 dunc homologue, Drosophila)	204735 at	212.51	121.629	1.747	+	84	0.001249
Inhibin α	210141 s at	475.26	212.213	2.24	+	85	0.0012506
TP53 target gene 1	209917 s at	278.896	739.962	0.377	−	86	0.0012653
Spermatogenesis-associated 2	204434 at	402.499	247.275	1.628	+	87	0.0012892
Insulin-induced gene 1	201626 at	1384.879	2681.165	0.517	−	88	0.0013424
Serum/glucocorticoid regulated kinase-like	220038 at	187.597	84.055	2.232	+	89	0.001394
Hypothetical protein LOC90333	214751 at	132.651	281.339	0.471	−	90	0.0014372
MRS2-like, magnesium homeostasis factor (*S. cerevisiae*)	218536 at	433.963	241.151	1.8	+	91	0.0014565
WNT inhibitory factor 1	204712 at	21.107	63.23	0.334	−	92	0.0014629
Polymerase (DNA directed) θ	219510 at	315.331	154.606	2.04	+	93	0.0014656
Ectonucleoside triphosphoate diphosphohydrolase 1	209474 s at	91.312	181.342	0.504	−	94	0.0015323
DKFZP586O0120 protein	201863 at	1066.931	2047.963	0.521	−	95	0.0015545
Cyclin D1	203003 at	71.257	27.906	2.553	+	96	0.0015729
Mouse mammary tumor virus receptor homologue 1	212484 at	1121.985	613.647	1.828	+	97	0.0016537
α Glucosidase II α subunit	211934 x at	853.545	1802.795	0.473	−	98	0.001746
Coiled-coil protein BICD2	213154 s at	356.548	616.454	0.578	−	99	0.0018189
KIAA0657 protein	212775 at	1599.555	879.879	1.818	+	100	0.001845
Bcl-2	207005 s at	104.988	26.786	3.92	+	101	0.0018496
Hypothetical protein DKFZp434G2311	212712 at	550.83	331.554	1.661	+	102	0.0018503
Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	202847 at	897.913	1530.352	0.587	−	103	0.0018568
Aldehyde oxidase 1	205082 s at	92.882	316.055	0.294	−	104	0.001889
KIAA0515	212069 s at	807.408	445.35	1.813	+	105	0.0019455
UDP-GlcNAc:betaGal β-1,3,-N-acetylglucosaminoyltransferase 6	203188 at	875.021	555.977	1.574	+	106	0.0019878
Platelet-derived growth factor receptor-like	205226 at	88.627	137.983	0.642	−	107	0.002008
c-src tyrosine kinase	202329 at	619.421	1025.285	0.604	−	108	0.0020142
Golgi autoantigen, golgin subfamily a, 2	35436 at	467.131	849.159	0.55	−	109	0.0020245
Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	201051 at	2169.058	1210.09	1.792	+	110	0.0020493
Hydroxy prostaglandin dehyrogenase 15-(NAD)	211549 s at	150.119	85.033	1.765	+	111	0.0021083
Hydroxy prostaglandin dehyrogenase 15-(NAD)	203914 x at	308.595	152.825	2.019	+	112	0.0021965
Butyrophilin, subfamily 3, member A3	204821 at	58.168	184.976	0.314	−	113	0.0022086
Galactokinase 2	205219 s at	240.849	388.952	0.619	−	114	0.0022089
Frequently rearranged in advanced T-cell lymphomas 2	209864 at	606.599	1012.928	0.599	−	115	0.0022226
Caldesmon 1	205525 at	29.357	46.41	0.633	−	116	0.0022346
Hydroxy prostaglandin dehydrogenase 15-(NAD)	203913 s at	255.449	117.952	2.166	+	117	0.0022542
Proteasome (prosome, macropain) subunit, β type, 8 (large multifunctional protease 7)	209040 s at	213.011	708.401	0.301	−	118	0.0023201
F-box and leucine-rich repeat protein 11	208988 at	544.302	942.391	0.578	−	119	0.0023575
Tara-like protein	210276 s at	268.181	592.816	0.452	−	120	0.002376
Myristoylated alanine-rich protein kinase C substrate	201670 s at	251.389	422.842	0.595	−	121	0.002406
Testis expressed sequence 27	218020 s at	1470.697	941.449	1.562	+	122	0.0024394
Suppression of tumorigenicity 7	207871 s at	128.766	226.667	0.568	−	123	0.0024562
Hemoglobin α 2	209458 x at	890.865	398.769	2.234	+	124	0.002482

siRNA concentration used in our experiments, 2×10^{-7} mol/L, produced a >80% reduction in cellular AIB1 protein levels (Fig. 1*B*). In addition, after a single transfection of siRNA, we found that the AIB1 protein levels were still repressed after 6 days (Fig. 1*C*). A control-scrambled sequence siRNA had no effect on AIB1 gene expression (Fig. 1*A–C*). The expression of a number of unrelated proteins such as actin or HER2 was unaltered by either siRNA. Thus, application of AIB1-directed siRNA was an effective and selective method of long-term suppression of endogenous AIB1 levels, enabling experiments to determine the impact of reducing endogenous AIB1 on MCF-7 cells.

Effect of Reduction of Endogenous AIB1 on Anchorage-independent Growth of MCF-7 Cells. An important growth factor induced phenotypic change, and a hallmark of malignant transformation is the ability of cells to form colonies in soft agar. This anchorage-independent growth involves cell cycle regulatory genes, as well as genes that prevent apoptosis under anchorage-independent conditions (*i.e.,* anoikis). IGF-I has been reported as a powerful inducer of anchorage-independent growth in MCF-7 breast cancer cells, and we first determined if AIB1 played a role in this IGF-I effect. IGF-I induced a 3-fold increase in the number of MCF-7 colonies formed, and this increase was completely negated by treatment with AIB1 siRNA (Fig. 2*A*). In contrast, a similar 3-fold increase in colony formation observed with serum treatment was unaffected by the reduction of AIB1 (Fig. 2*A*), suggesting a selective role of AIB1. In 1% CCS, the colony count is indistinguishable from the baseline of this assay, and this is unaffected by AIB1 siRNA treatment. The effects of AIB1 siRNA on IGF-I–induced colony formation were not

due to nonspecific activation of RNA degradation because parallel experiments in which endogenous AIB1 were reduced by the expression of tetracycline-regulated ribozymes (14) showed the same selective inhibition of IGF-I–induced colony formation (data not shown).

Increases in colony formation in anchorage-independent growth due to growth factor stimulation result from a balance between increased numbers of cells entering the cell cycle and reduction in anoikis. Therefore, it was of interest to determine which of these processes was affected by the reduction of AIB1. To examine this, we grew MCF-7 cells on poly-HEMA–coated dishes that prevent attachment and forces the cells to grow anchorage independently (24). Fluorescence-activated cell sorting analysis showed that reduction of AIB1 levels had no significant effect on progression of the cells into G_2 + S either under basal, IGF-I, or serum-induced growth conditions (Table 1*A*). Annexin V staining to quantitate apoptosis, however, showed that IGF-I was unable to rescue the cells with reduced AIB1 levels from anoikis, whereas the rescue by serum was unaffected (Fig. 2*B*). The increase in anoikis in 1% CCS in the presence of AIB1 siRNA did not register in the soft agar colony count because this was already at background levels of this latter assay in the absence of AIB1 siRNA treatment and is therefore not sensitive to additional reductions. Thus, there are no factors in 1% CCS that stimulate colony formation, but AIB1 siRNA can still cause apoptosis in these cells because there are no factors in 1% CCS to rescue them. Overall, the results suggest that IGF-I–induced survival of cells growing in suspension depends on an AIB1-dependent signaling pathway, whereas serum does not.

AIB1 Effects on Anchorage-dependent Growth. To determine whether the effect of AIB1 siRNA were unique to anchorage-independent growth conditions, we also determined its effects under anchorage-dependent growth conditions. Attached cells show a reduced growth rate (~30%) after reduction of AIB1 and the stimulation by IGF-I (1.7-fold) or by serum (2.8-fold) was not affected by reduced AIB1 levels. These data imply that cell attachment can circumvent the rate-limiting signal pathways required for IGF-I–dependent survival of cells in suspension (Fig. 3*A*). Interestingly, AIB1 siRNA treatment reduced by 34% the number of MCF-7 cells in G_2 + S phases of the cell cycle (Table 1*B*), which explains the reduction of the overall growth rate independent of exogenously added growth stimulus. Under attached growth conditions, there was a trend to increased apoptosis with reduction in AIB1 with siRNA, but the differences were not statistically significant (Fig. 3*B*). In the MCF-7 Rz29 cell lines (14), similar results were also obtained (data not shown).

Analysis of Genes Whose Expression Is Dependent on AIB1. To determine genes that might be critical targets of AIB1 during proliferative responses in MCF-7 cells, we compared gene array analysis of MCF-7 cells after treatment with AIB1 or control siRNA for 2 days. The Affymetrix U133A human gene chip that represents 33,568 transcripts was used to evaluate gene expression profiles under these different conditions, and we found 124 genes that showed a statistically significant difference between AIB1 high (control siRNA transfected) and AIB1 low (AIB1 siRNA transfected) at $P \leq 0.0025$ (Table 2). Interestingly, several genes that are known to be critical for cell cycle regulation, anoikis, and apoptosis, notably cyclin D1, Bcl-2, and MAPK [extracellular signal-regulated kinase (ERK) 1/2] were highly dependent on AIB1 levels for their sustained expression (highlighted in Table 2). In our array analysis, we also found that the expression of a number of genes was up-regulated by reducing AIB1 with siRNA (Table 2), indicating that high AIB1 levels normally suppress expression of these genes.

In a separate set of array analyses, we also examined the effect of a short-term 1 hour treatment by IGF-I on gene expression after treatment of MCF-7 cells with AIB1 siRNA and control siRNA. This was to determine whether there were critical immediate early genes that IGF-I–regulated dependent on the AIB1 levels. However, at $P \leq 0.0025$, this analysis did not differ significantly from the AIB1 siRNA *versus* control siRNA analysis. Thus, 1 hour of IGF-I treatment after pretreatment with AIB1 siRNA did not alter gene expression in comparison to cells pretreated with AIB1 siRNA alone (this comparison is not shown).

To assess whether altered mRNA expression due to AIB1 reduction was also reflected at the protein level, we did a series of Western blot analysis for those proteins known to be crucial for cell growth and survival. The protein levels of cyclin D1, Bcl-2, and ERK2 (but not ERK1) were reduced (90, 40, and 40%, respectively) by lowering the AIB1 levels by >90% in attached MCF-7 cells (Fig. 4). AIB1 siRNA reductions in expression of these genes were also observed in the presence of IGF-I (Fig. 4). IGF-I clearly induced the expression of cyclin D1 (4-fold) and caused a smaller 1.7-fold increase in the protein expression of ERK2 (Fig. 4). The basal levels of both cyclin D1 and Bcl-2 were increased under anchorage-independent conditions (Fig. 4), and IGF-I did not increase these levels any further (Fig. 4). Overall, it is clear that in basal or IGF-I–treated conditions or in cells attached or in suspension, targeting of AIB1 was effective in producing decreases in cyclin D1, Bcl-2, and ERK2 protein levels. Maintenance of the expression of these genes by AIB1 could be involved in both basal and IGF-I–induced growth responses in MCF-7 cells.

AIB1 Is Required to Maintain Expression of Molecules Critical to Insulin-like Growth Factor I Signaling. A somewhat surprising aspect of our cDNA array analyses was that after 48 hours of exposure to AIB1 siRNA, we did not see significant changes in the mRNA levels of molecules that specifically transmit IGF-I signaling such as the IGF-IR, IRS-1, IRS-2, or IGF-binding protein family members. We had conjectured that changes in the expression of these molecules after reduction of AIB1 might explain the selective effect on IGF-I but not serum signaling, and thus, we next determined the impact of the reduction of AIB1 on the protein levels of IGF-I–signaling molecules to pick up potential posttranscriptional effects. We did Western blot analysis of IGF-I–signaling molecules in the presence of control or AIB1 siRNA in both attached and suspension growth (Fig. 5, left and right panels). Under the anchorage-independent conditions, which had revealed the dependence of IGF-I survival signals on AIB1 (see

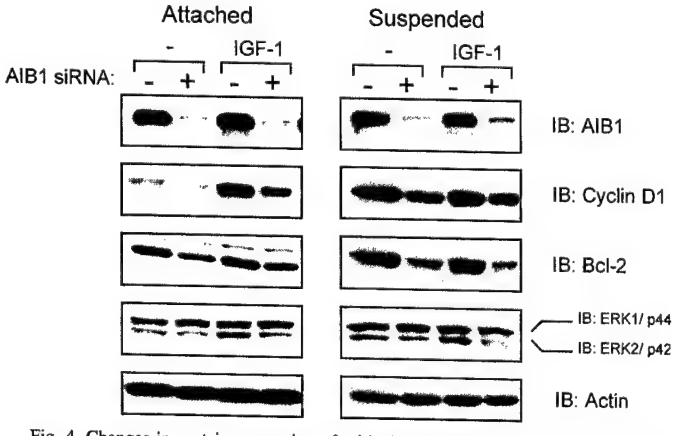

Fig. 4. Changes in protein expression of critical genes involved in proliferation, cell cycle, and apoptosis after AIB1 siRNA treatment. For attached cells, estrogen-stripped MCF-7 cells were transfected with control or AIB1 siRNA and treated with or without IGF-I in the presence of 1% CCS. Whole cell lysates were harvested after 48 hours and analyzed by Western blot analysis. In suspension growth conditions, cells were transfected for 18 to 24 hours with siRNA and replated in poly-HEMA (10 μg/mL)–coated dishes for another 24 hours before whole cell lysates were harvested and analyzed by Western blot analysis. Data in Fig. 6 are from the same experiment as Fig. 4. Blots are representative results from three independent experiments.

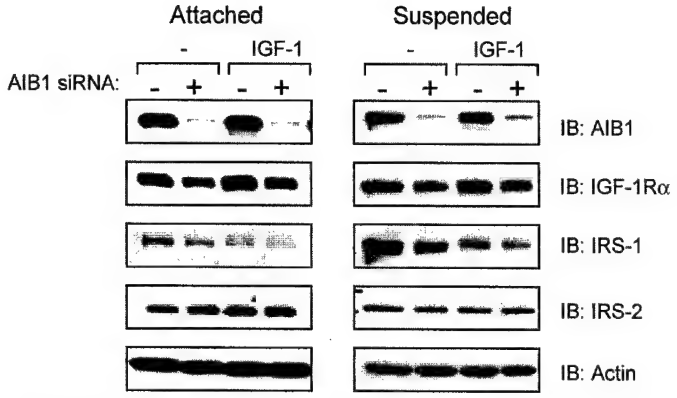

Fig. 5. AIB1 has a role in regulating some members of the IGF-I–signaling pathway. For attached cells, estrogen-stripped MCF-7 cells were transfected with control or AIB1 siRNA and treated with or without IGF-I in the presence of 1% CCS. Whole cell lysates were harvested after 48 hours and analyzed by Western blot analysis. In suspension growth conditions, cells were transfected for 18 to 24 hours with siRNA and replated in poly-HEMA (10 μg/mL)–coated dishes for another 24 hours before whole cell lysates were harvested and analyzed by Western blot analysis. Blots are representative results from three experiments.

Fig. 2), we found that IGF-I receptor protein levels were reduced by 50% upon reduction of AIB1. Furthermore, we also observed a smaller 10 to 20% reduction in IRS-1 protein levels in the presence of IGF-I (Fig. 5*A*, right panel). IRS-2 protein was not altered. IGF-IR levels in attached cells showed a similar reduction of the IGF-I receptor protein after AIB1 siRNA treatment and no detectable alteration in IRS-1 or IRS-2 proteins (Fig. 5, left panel). Thus, we conclude that the IGF-I receptor is dependent on AIB1 for its protein expression levels in MCF-7 cells. This regulation appears to be mediated at the posttranscriptional level because we did not detect significant changes in the IGF-IR mRNA levels on the cDNA array analysis.

AIB1 Is Required for Estrogen-dependent and -independent Regulation of Gene Expression in MCF-7 Cells. We have determined previously that AIB1 plays a rate-limiting role in the estrogen-induced proliferation of MCF-7 cells (14). There is much evidence for cross-talk between the IGF-I and ER-signaling pathways (25–27) and the expression of IGF-1R, IRS-1, cyclin D1, Bcl-2, and MAPK are all regulated by estrogen (28–33). We therefore hypothesized that some if not all of the effects that we saw on gene expression by reducing cellular levels of AIB1 might be mediated through a reduction in ER signaling. Interestingly, we found that the expression of ER-α itself was not reduced under basal conditions after AIB1 siRNA treatment under attached conditions (Fig. 6*A*). However, in suspension conditions, we observed a decrease in ER levels in the presence of AIB1 siRNA. Thus, it was possible that the ER reduction alone could explain some of the effects of AIB1 siRNA in suspension. To examine the role of estrogen in the gene expression changes that we observed in the presence of AIB1 siRNA, we repeated the analysis of protein expression changes in the presence of the antiestrogen ICI 182,720 (ICI, Faslodex), which blocks estrogen binding to the receptor, as well as down-regulates the ER levels (Fig. 6*A*, right panel; ref. 34). We argued that if the effects of AIB1 were dependent on ER, then in the presence of antiestrogen, we would no longer see the AIB1 siRNA effect. Interestingly, AIB1 siRNA-dependent reductions in cyclin D1, Bcl-2, and ERK2 that we had observed in Fig. 4 were all preserved in the presence of ICI under attached conditions (Fig. 6*B*). However, the basal reduction in cyclin D1 was only 30% compared with the >80% reduction that we saw in the absence of ICI. This suggests that a large portion of the basal expression of cyclin D1 is maintained through estrogen signaling. In contrast, the magnitude of the AIB1 siRNA reduction in Bcl-2 and ERK2 were comparable in the presence or absence of antiestrogen. Although the IGF-IR is known to be an estrogen-induced gene, AIB1 siRNA treatment was still able to decrease IGF-IR expression irrespective of ICI (compare Fig. 5 with Fig. 6*B*). Overall, this data indicates that a large portion of the basal and IGF-I–induced changes in gene expression that we observed that were dependent on AIB1 were occurring independent of ER signaling.

AIB1 Is Required for IGF-I Signaling and Phenotypic Changes in the MDA MB-231 Breast Cancer Cell Line. To determine whether the dependence of IGF-I signaling was observed in other breast cancer cell lines, we examined the effect of AIB1 siRNA on the human breast cancer cell line, MDA MB-231. These cells are ER negative and proliferate in response to IGF-I (28). To determine whether similar genes were involved in the AIB1 effect in these cells as MCF-7 cells, we did immunoblot analysis of cell cycle and apoptosis genes, as well as for molecules involved specifically in IGF-I signaling. Similar to MCF-7 cells, cyclin D1 and ERK2 gene expression were dependent on maintaining high AIB1 levels (Fig. 7*A*). However, Bcl-2 levels were unaffected by AIB1 siRNA treatment. Unlike MCF-7 cells, none of these genes were induced by IGF-I in the MDA MB-231 cells (Fig. 7*A*). Interestingly, in MCF-7 cells, we observed that IGF-IR and to a certain extent IRS-1 expression were dependent on AIB1, but in MDA MB-231 cells, no changes in the expression of any of these proteins was observed (Fig. 7*B*).

The Role of AIB1 in IGF-I–induced Phosphatidylinositol 3′-Kinase (PI3k) and MAPK Signaling in Breast Cancer Cells. The data thus far indicated that neither the estrogenic effects nor the regulation of IGF-I–signaling molecules was the principal mechanism whereby AIB1 specifically effected IGF-I signaling. We therefore sought to de-

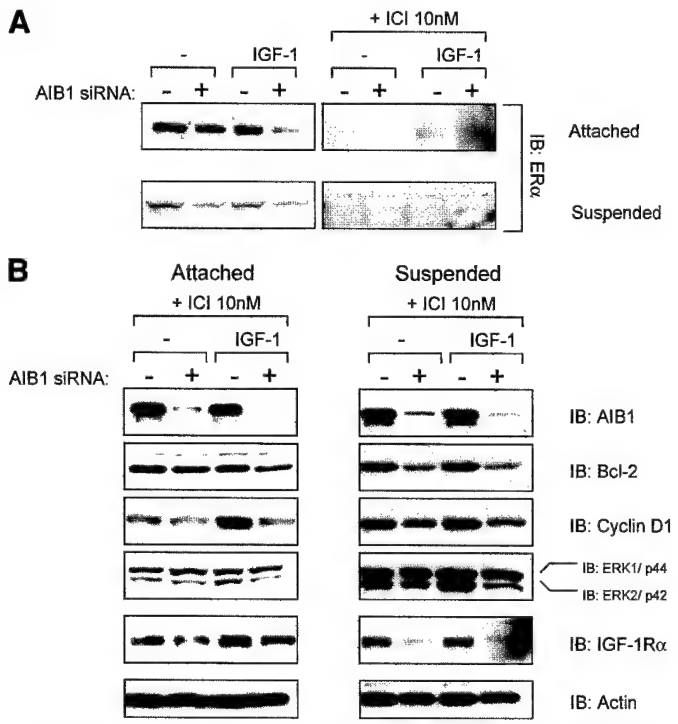

Fig. 6. The ability of AIB1 to regulate the expression of Bcl-2, cyclin D1, IGF-IR, and MAPK in the presence of IGF-I is independent of estrogen signaling. *A* and *B*. In attached growth conditions, estrogen-stripped MCF-7 cells were transfected with control or AIB1 siRNA and treated with or without IGF-I in the presence of 1% CCS and 10 nmol/L ICI. Whole cell lysates were harvested after 48 hours. In suspension growth conditions, cells were transfected for 18 to 24 hours with siRNA and replated in poly-HEMA (10 μg/mL)–coated dishes for another 24 hours before whole cell lysates were harvested. Lysates were analyzed by Western blot analysis with antibody probes as indicated. Data in Fig. 6 are from the same experiment as Fig. 4. Blots are representative results from three experiments.

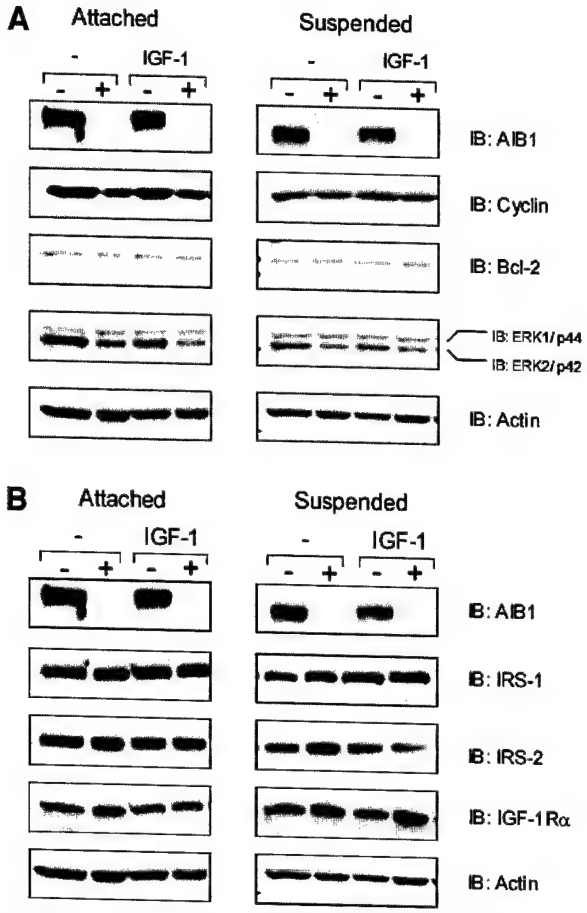

Fig. 7. Reduction of AIB1 in an ER-α–negative breast cancer cell line, MDA MB-231, regulates the expression of cyclin D1. *A* and *B*. In attached growth conditions, MDA MB-231 cells were transfected with control or AIB1 siRNA and treated with or without IGF-I in the presence of 1% CCS. Whole cell lysates were harvested after 48 hours. In suspension growth conditions, cells were transfected for 18 to 24 hours with siRNA and replated in poly-HEMA (10 μg/mL)–coated dishes for another 24 hours before whole cell lysates were harvested. Lysates were analyzed by Western blot analysis with antibodies as indicated. Blots are representative results from two experiments.

termine whether down-regulation of AIB1 directly affected activation of IGF-I–signaling pathways. For these experiments, we pretreated MCF-7 or MDA MB-231 cells (attached or suspended) for 48 hours with AIB1 siRNA and then determined the responsiveness of the cells to short-term treatment with IGF-I (0 to 30 minutes; Fig. 8). We examined the levels of AKT and its phosphorylation status on Ser^{473} as a readout of the sensitivity of the PI3k pathway and measured ERK 1/2 levels and their phosphorylation as a read out of MAPK activation. In attached conditions, in both MCF-7 and MDA MB-231 cells, we observed a reduction in the ratio of phospho-AKT to AKT, indicating a decreased sensitivity of the PI3k pathway to IGF-I (Fig. 8, *A* and *B*). However, the reduction in MCF-7 cells was less than that observed in MDA MB-231 cells, and the reduction in the phospho-AKT to AKT ratio was primarily due to an increase in AKT levels rather than a reduction in phosphorylation of AKT (Fig. 8A). Under suspension conditions (equivalent to the soft agar colony formation conditions) the IGF-I–induced changes in phospho-AKT to AKT ratio are unaltered in MCF-7 cells by AIB1 siRNA treatment, but the ratio was still reduced in MDA MB-231 cells (Fig. 8, *C* and *D*).

In contrast to the changes in AKT activation under all conditions and in both cell lines, treatment with AIB1 siRNA did not change the time course or the magnitude of ERK 1/2 phosphorylation after IGF-I stimulation (Fig. 8*A–D*). Overall, these data indicate that AIB1 levels determine the sensitivity of the PI3k pathway to IGF-I signaling in these breast cancer cell lines under certain plating conditions, but changes in AIB1 levels appear to have little effect on MAPK signaling.

DISCUSSION

The role of AIB1 and other coactivators in nuclear receptor signaling is well established. In this study, we now show that AIB1 can also be rate-limiting for IGF-I–stimulated growth of MCF-7 human breast tumor cells. This is consistent with the finding in p/CIP (AIB1 mouse homologue)-knockout mice that IGF-I stimulation of proliferation of mouse embryonic fibroblast cells was significantly reduced by deletion of p/CIP (18). However, in contrast to Wang *et al.* (18), where only a few changes in gene expression were observed upon array analysis of $pCIP^{-/-}$ *versus* wild-type mouse embryonic fibroblast cells, in MCF-7 cells, a number of critical signaling molecules were dependent on AIB1 for their expression and regulation. Most significantly altered was cyclin D1, whose expression was highly dependent on AIB1 expression under anchorage-dependent and -independent conditions. Cyclin D1 is an important cell cycle-regulating protein that in response to mitogenic stimulus associates with CDK4/6. The cyclin D1-CDK4/6 complex hyperphosphorylates the retinoblastoma protein, leading to the release of E2F. Free from its association with the retinoblastoma protein, E2F activates genes necessary for cell proliferation (35). Cyclin D1 is a critical downstream target of PI3k-signaling pathway for proliferation in MCF-7 cells (36). It is likely that the reduction in cyclin D1 under low AIB1 conditions would explain some of the cell cycle effects of AIB1 siRNA treatment of MCF-7 cells under anchorage-dependent conditions. However, it appears that the cells are less dependent on cyclin D1 for cell cycle control under anchorage-independent conditions. Our data confirm and extend previous observations that cyclin D1 gene regulation by estrogen is AIB1 dependent (37). In addition, we show that a portion of the basal expression of cyclin D1, as well as its IGF-I induction, occurs independent of estrogen signaling but is nevertheless AIB1 dependent (Fig. 6*B*).

The antiapoptotic protein Bcl-2 was also found in our cDNA array study to be significantly down-regulated in the absence of AIB1. Bcl-2 is a proto-oncogene that is a part of a family of related proteins that functions to either promote or inhibit apoptosis and anoikis (38, 39). In the presence of AIB1 siRNA, Bcl-2 levels were decreased in both basal and IGF-I treatment conditions, regardless of attached or poly-HEMA growth conditions. Bcl-2 is thought to play a central role in the interplay of integrin and IGF-I signaling in anoikis (40, 41), and reductions in Bcl-2 may explain some of the abrogation of the anti-anoikis effect of IGF-I in suspension. Interestingly, Bcl-2 and cyclin D1 expression are interconnected. When Bcl-2 was overexpressed in a human breast epithelial cell line, MCF10A, it caused an increase in the expression of cyclin D1. Bcl-2 was also found to be able to increase the activity of cyclin D1 promoter in both MCF10A and MCF-7 cells independent of anchorage conditions (42). The effects of both the loss of Bcl-2 and AIB1 could therefore contribute to greater reduction in cyclin D1 levels.

Our data indicate that a large portion of the transcriptional control of genes, such as cyclin D1 and Bcl-2, occurs independent of the ER. AIB1 is known to coactivate a number of factors that are not nuclear receptors such as NF-κB (43, 44) and activator protein-1 (21). It is likely that some of these sites are in involved in AIB1 control of basal and IGF-I control of gene transcription in MCF-7 cells. Of note is that both cyclin D1 and Bcl-2 genes are estrogen-inducible genes that contain nonclassical ERE promoters because they do not contain half or full consensus ERE sites in their promoters. Both genes contain in common cAMP-response element, activator protein-1, and Sp-1 sites, which are necessary for estrogen induction of the promoter (29, 33, 45). Furthermore, IGF-I induction of the Bcl-2 promoter is dependent on a cAMP-response element site (46). Thus, it is possible that AIB1 may act at the cAMP-response element, activator protein-1, or Sp-1

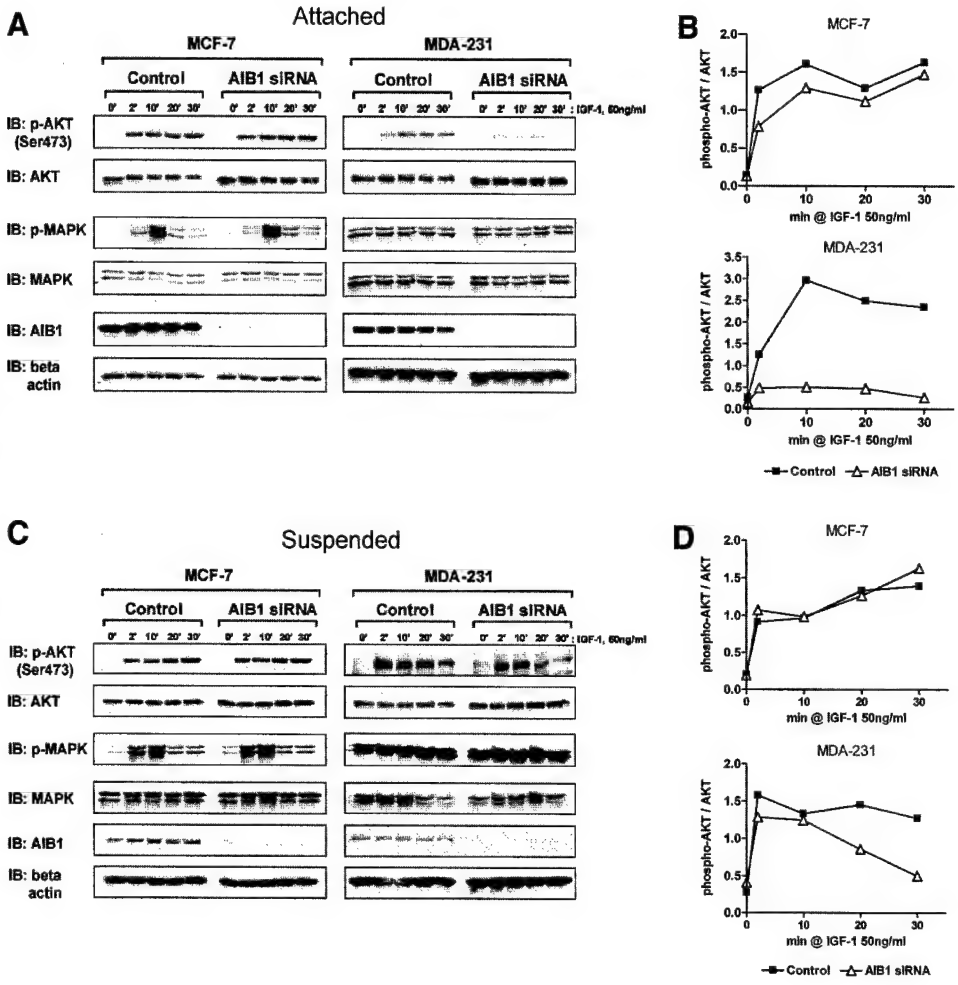

Fig. 8. Role of AIB1 for IGF-I induced AKT and MAPK phosphorylation. *A*. In attached conditions, estrogen-stripped MCF-7 and MDA MB-231 cells were transfected with control or AIB1 siRNA for 48 hours and treated with 50 ng/mL IGF-I. Whole cell lysates were harvested at the indicated time points. *C*. In suspension growth conditions, estrogen-stripped cells were transfected for 18 to 24 hours with siRNA and replated in poly-HEMA (10 μg/mL)–coated dishes for another 24 hours before IGF-I treatment. Whole cell lysates were harvested at the indicated time points. Relative band intensities were assessed using densitometry. Densitometry values were used to calculate the ratio between phosphorylated AKT and total levels of AKT and are represented as line graphs in *B* and *D*.

sites to coactivate expression in the presence of IGF-I, which may not necessitate the presence of the ER.

Although the changes in cyclin D1, Bcl-2, and MAPK could explain the effects of AIB1 siRNA on basal proliferation, it is not clear that these changes would specifically affect the IGF-I *versus* serum pathway. In the analysis of the mouse embryonic fibroblast p/CIP$^{-/-}$ cells, no real changes were observed in IGF-I–signaling molecule pathway molecules (18). Our study of MCF-7 cells indicates that AIB1 plays a role in controlling levels of IGF-IR, and this is especially pronounced under anchorage-independent conditions. In MDA MB-231 cells, however, the IGF-IR levels were unaltered by changes in AIB1 levels and thus do not appear to be involved in AIB1 control of IGF-I proliferation in these cells. As stated above, IGF-IR interplay with the integrin-signaling pathways plays a major role in the control of anoikis in many cell types (47–49), and it appears that AIB1 may be able to potentiate IGF-I inhibition of anoikis in MCF-7 cells by maintaining high IGF-IR levels. Importantly, the maintenance of the high IGF-IR levels occurred even when ER signaling was reduced, suggesting that AIB1 could maintain this phenotype even when breast cancer cells are growing hormone independently. Changes in IRS-1 levels have been observed to be AIB1 dependent in H-*ras*–induced tumors (50), but the decreases in IRS-1 we observed with AIB1 siRNA were small in MCF-7 cells and not observed in the MDA MB-231 cells.

A recent article by Zhou *et al.* (51) found that overexpressing AIB1 caused an increase in AKT levels and an increase in cell size in prostate cancer cells in a steroid-independent manner. In MCF-7 or MDA MB-231 cells, we did observe some changes in unphosphorylated AKT levels but did not observe changes in cell size when AIB1 was down-regulated. Differences in basal AKT regulation between cell types might be explained by differences in PTEN gene status in the cell lines used in these studies. The prostate cancer cell lines, LNCaP and PC-3, used in the Zhou *et al.* (51) study have a defective PTEN gene (52), whereas MCF-7 and MDA MB-231 breast cancer cells have a wild-type PTEN gene (53). It does seem clear from our data that the ability of IGF-I to activate AKT is dependent on AIB1 levels in these breast cancer cell lines at least under certain plating conditions. However, the most profound AIB1-dependent phenotypic change induced by IGF-I is observed when MCF-7 cells are growing anchorage independently. Recall that under these conditions we see little change in the IGF-I activation of AKT, thus it seems likely that AIB1 is also involved in other signaling pathways that predominate when the cells are growing in suspension.

In conclusion, our results suggest that IGF-I induced survival of cells in suspension depends on AIB1 signaling whereas the serum does not. Also, some IGF-I–induced changes in gene expression are dependent on AIB1. The observation that AIB1 is rate-limiting for aspects of IGF-I signaling is important because it implies that AIB1 could effect malignant phenotypic changes induced by growth factors, even if estrogen signaling is not intact. This may become relevant in advanced breast cancer, which is frequently estrogen receptor negative, but has amplified growth factor signaling from the IGF-I–signaling pathways (54). In addition, overexpression of AIB1 may play a role in potentiating growth factor signaling when estrogen signaling is blocked with antiestrogens, thus contributing to the antiestrogen-resistant phenotype in breast cancer. Our studies suggest a broader role of AIB1 in coactivating growth signaling that is not limited to hormonally responsive breast tissue but may be extended to

other tissues that are responsive to IGF-I such as the pancreas (55) and prostate (56).

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An Isoform of the Coactivator AIB1 That Increases Hormone and Growth Factor Sensitivity Is Overexpressed in Breast Cancer*

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The AIB1 (amplified in breast cancer 1) protein is a coactivator that potentiates the transcriptional activity of nuclear hormone receptors, and its gene is amplified in a subset of human breast cancers. Here we report a splice variant of *AIB1* mRNA that lacks the exon 3 sequence. We determined that the *AIB-Δ3* mRNA encoded a 130-kDa protein that lacks the NH_2-terminal basic helix-loop-helix and a portion of the PAS (Per-Arnt-Sim homology) dimerization domain. The 130-kDa protein was detected in MCF-7 breast cancer cells at levels that were 5–10% of the full-length protein, whereas in nontransformed mammary epithelium lines, the AIB-Δ3 protein was present at significantly lower levels compared with the full-length AIB1. Consistent with this finding, the abundance of *AIB1-Δ3* mRNA was increased in human breast cancer specimens relative to that in normal breast tissue. To determine whether there were phenotypic changes associated with the overexpression of the AIB-Δ3 isoform, we performed functional reporter gene assays. These revealed that the ability of AIB1-Δ3 to promote transcription mediated by the estrogen or progesterone receptors was significantly greater than that of the full-length protein. Surprisingly, the AIB1-Δ3 isoform was also more effective than AIB1 in promoting transcription induced by epidermal growth factor. Overexpression of AIB1-Δ3 may thus play an important role in sensitizing breast tumor cells to hormone or growth factor stimulation.

Ligands such as estrogen and progesterone that interact with nuclear receptors regulate gene expression predominantly at the transcriptional level. The ligand-bound receptors interact specifically with DNA and activate transcription by recruiting a preinitiation complex. Although such gene activation was originally thought to be mediated by interaction of the receptors with components of the basal transcriptional machinery (1–6), a variety of screening techniques has identified a family of receptor-interacting proteins known as nuclear receptor coactivators (7–11). A common characteristic of this superfamily of proteins is that, when overexpressed in the presence of nuclear receptors, they potentiate ligand induction of transcription (12, 13). Members of the related p160 group of coactivators, which include steroid receptor coactivator 1 (SRC-1),[1] SRC-2, and SRC-3 (also known as AIB1, ACTR, RAC3, TRAM-1, and p/CIP) (14–20), possess several similar structural features including a receptor interaction domain, a bHLH (basic helix-loop-helix)-PAS (Per-Arnt-Sim homology) dimerization domain, and a CBP interaction domain (13). Coactivators are thought to function as bridges between nuclear receptors and either other coactivators or the basal transcriptional machinery (13). However, the discovery that coactivators possess a histone acetylase domain (15, 21–24) suggests that these proteins also might serve to regulate chromatin structure.

A portion of human chromosome 20q that is frequently amplified in breast cancer contains the gene for the nuclear coactivator AIB1 (amplified in breast cancer 1) (25). The *AIB1* gene is amplified in 5–10% of breast cancers, and the abundance of the corresponding mRNA and protein is increased in some breast tumors and breast cancer cell lines (14, 25–27). It has recently been shown that AIB1 binds directly to the estrogen receptor (ER) (28) and that AIB1 is rate-limiting for estrogen-induced growth of MCF-7 cells (29). However, the overall role of AIB1 for breast tumorigenesis is not clear because AIB1 potentiates not only the action of estrogen (14, 16) and progesterone (16) receptors but also that of various other nuclear receptors (9, 15, 17–20) and transcription factors (30, 31). In addition, several splice variants of SRC family members have been described, although the functions of these variants remain unknown (13).

Here we report the identification of a splice variant of *AIB1* that is overexpressed in breast cancer tissue and cell lines. The *AIB-Δ3* transcript encodes an NH_2-terminal truncated version of AIB1 that lacks the bHLH and PAS A domains. In functional studies we have determined that the AIB-Δ3 protein is a significantly more effective coactivator of estrogen, progesterone, and EGF signaling than the wild type ER, suggesting a role for this AIB1 isoform in hormone and paracrine signaling in breast cancer.

EXPERIMENTAL PROCEDURES

Plasmids—We subcloned the full-length *AIB1* cDNA from pCMX-ACTR (provided by R. Evans, Salk Institute, La Jolla, CA) into pcDNA3 (Invitrogen) with the use of the flanking *Kpn*I and *Xho*I sites, thereby creating the expression vector pcDNA3-AIB1. We subcloned the smaller of the two RT-PCR products generated from MCF-7 cell total RNA with exon 1- and exon 9-specific primers (Fig. 1*b*) into pCRII (Invitrogen). The resulting plasmid was digested with *Bam*HI and *Hpa*I, recognition sequences that flank the splice sites of *AIB1-Δ3* cDNA, and the released fragment was purified and used to replace the corresponding sequence of pcDNA3-AIB1, thereby creating pcDNA3-AIB1-Δ3. The pcDNA3-

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[1] The abbreviations used are: SRC, steroid receptor coactivator; bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim homology; ER, estrogen receptor; ERE, estrogen response element; PCR, polymerase chain reaction; RT, reverse transcription; IMEM, Iscove's modified Eagle's medium; FBS, fetal bovine serum; EGF, epidermal growth factor; CHO, Chinese hamster ovary; FGF-BP, fibroblast growth factor-binding protein; TEF, transcription-enhancing factor.

AIB1 and pcDNA3-AIB1-Δ3 vectors contain identical 5′- and 3′-untranslated regions, differing only in the loss of exon 3 in the latter. The inserts were verified by sequencing.

The expression vectors for human estrogen receptor α and progesterone receptor B were provided by P. Chambon (CNRS, France). The firefly luciferase reporter plasmid containing the estrogen response element (ERE) from the *Xenopus* vitellogenin gene was provided by V. C. Jordan (Northwestern University, Chicago), and the plasmid containing the mouse mammary tumor virus promoter was provided by G. Hager (National Cancer Institute, Bethesda, MD). The luciferase reporter plasmid containing the human *FGF-BP* gene promoter has been described previously (32). The *Renilla* luciferase vector (pRL-CMV) was from Promega.

Cells and Tissue Samples—All cell lines were obtained from the tissue culture core facility of the Lombardi Cancer Center. MCF-7, ME-180, and COS-1 cells were cultured in Iscove's modified Eagle's medium (IMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). MCF-10A and A1N4 cells were grown in a 1:1 mixture of IMEM and Ham's F-12 medium (Life Technologies, Inc.) that was supplemented with 5% horse serum, EGF (20 ng/ml), insulin (10 μg/ml), and hydrocortisone (500 ng/ml). CHO cells were maintained in F-12 nutrient mixture (Life Technologies, Inc.) supplemented with 10% FBS.

Frozen tissue samples were obtained from the Lombardi Cancer Center Histopathology and Tissue Shared Resource Core. The six normal samples were obtained from individuals undergoing reduction mammoplasty (mean age at time of surgery, 29 years; range, 19 to 54 years); the eight primary breast carcinoma specimens were obtained from women with a mean age at the time of surgery of 51 years (range, 29 to 64 years).

Immunoblot Analysis—Whole cell extracts were prepared as described previously (32), and equal portions (30 μg of protein) were resolved either on denaturing 4–20% polyacrylamide gradient gels or on 4% polyacrylamide gels containing Tris-glycine. The separated proteins were transferred to a nitrocellulose membrane and then subjected to immunoblot analysis with a 1:500 dilution of a mouse monoclonal antibody specific for amino acids 376–389 of human AIB1 (Transduction Laboratories), horseradish peroxidase-conjugated goat antibodies to mouse immunoglobulin (1:10,000 dilution; Amersham Pharmacia Biotech), and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

RT-PCR—Isolation of total RNA and synthesis of cDNA by RT were performed as described previously (33). The amplification of *AIB1* cDNA sequences was achieved by PCR according to the following protocol: incubation at 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 90 s. The oligonucleotides used as primers for PCR or as probes for hybridization were as follows: exon 1, 5′-GACTGGTTAGCCAGTTGCTG-3′; exon 2, 5′-GCCATGTGATACTCCAGGAC-3′; exon 3, 5′-CTGAGCTGATATCTGCCAATC-3′; exon 4, 5′-AGCCGATGTATCTTCTACAGG-3′; exon 5, 5′-ATGTTTCCGTCTCGATTCACC-3′; exon 8, 5′-CCTCATGGAGGATCTCAGTG-3′; and exon 9, 5′-CCATCAGCCAACGAGAATCG-3′. The PCR products were separated by electrophoresis on a 1% agarose gel, transferred to a polyvinylidene difluoride membrane, and hybridized with a ^{32}P-labeled oligonucleotide probe. Quantification of PCR products was performed with a PhosphorImager (Molecular Dynamics 445SI).

Transient Transfection and Reporter Gene Assays—COS-1 and CHO cells were plated at densities of 2×10^5 and 5×10^5 cells/well, respectively, in six-well plates and were cultured for 24 h at 37 °C under 5% CO_2 in IMEM or Ham's F-12, respectively, supplemented with 5% FBS that had been treated with dextran-coated charcoal. The medium was then replaced with IMEM containing LipofectAMINE Plus (Life Technologies, Inc.) and expression and reporter plasmids as indicated. After incubation for 3 h, the medium was replaced with IMEM (COS-1 cells) or Ham's F-12 (CHO cells), each containing 5% dextran-coated charcoal-treated FBS and nuclear receptor ligands. Cells were incubated for 24 h and then disrupted in passive lysis buffer (Promega). Portions (20 μl) of the resulting cell extract were assayed for both firefly and *Renilla* luciferase activities with the Dual-Luciferase reporter assay system (Promega).

ME-180 cells were plated at a density of 5×10^5 cells/well and cultured for 24 h in IMEM supplemented with 5% dextran-charcoal-treated FBS. They were then incubated for 3 h in IMEM supplemented with LipofectAMINE Plus and expression and reporter plasmids. The cells were washed and then incubated in IMEM for an additional 3 h before incubation for 18 h with EGF (5 ng/ml) in serum-free IMEM and subsequent lysis. Because of high background induction of pRL-CMV expression by EGF, firefly luciferase activity was normalized by protein concentration as described previously (32).

In Vitro Transcription-Translation—*In vitro* transcription-translation was performed with the TnT coupled reticulocyte lysate system (Promega). Plasmid DNA (1 μg) was combined with 25 μl of rabbit reticulocyte lysate, 2 μl of TnT reaction buffer, 1 μl of T7 RNA polymerase, 1 μl of amino acid mixture, 1 μl of Rnasin (Ambion) ribonuclease inhibitor (40 units), and 1 μl of Transcend biotin-lysyl-tRNA (Roche) and the final volume was adjusted to 50 μl. The reaction was performed at 30 °C for 90 min, after which 5 μl of the reaction mixture was subjected to SDS-polyacrylamide gel electrophoresis and either to immunoblot analysis with antibodies to AIB1 or to direct detection with streptavidin-conjugated horseradish peroxidase (1:10,000 dilution in phosphate-buffered saline containing 0.05% Tween 20) and enhanced chemiluminescence.

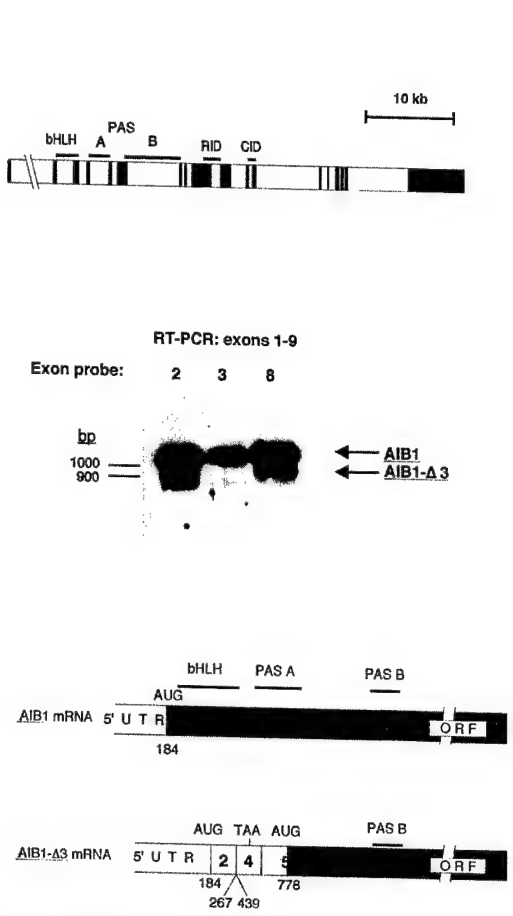

FIG. 1. **Characterization of a splice variant of human *AIB1*.** *a*, structure of human *AIB1* showing the 22 known exons (*filled boxes*) and the corresponding introns (*open boxes*). The exon/intron regions, which are spliced to form the various functional domains of the AIB1 protein, are indicated by *horizontal bars*. *b*, detection of the *AIB1-Δ3* splice variant in total RNA from MCF-7 cells. The total RNA was subjected to RT-PCR with primers specific for exons 1 and 9 of *AIB1*. The reaction products were resolved on a 1% agarose gel and transferred to a polyvinylidene difluoride membrane, which was then cut, and the lanes were subjected separately to hybridization with ^{32}P-labeled oligonucleotides specific for exons 2, 3, or 8 of *AIB1*. The positions of PCR products corresponding to the full-length (*AIB1*) and truncated (*AIB1-Δ3*) transcripts are indicated. *bp*, base pairs. *c*, comparison of the structures of *AIB1* and *AIB1-Δ3* mRNAs. The alternative splicing event that results in the loss of exon 3 causes the open reading frame (*ORF*) to shift and terminate at a TAA codon in exon 4. A potential initiation site (*AUG*) for *AIB1-Δ3* mRNA is present at nucleotide 778; the use of this site would be consistent with the AIB-Δ3 protein lacking the NH_2-terminal 26 kDa of full-length AIB1. The *shaded* regions indicate the open reading frame, and exons in the mRNAs are *numbered*. The positions of the splice junctions in *AIB1-Δ3* mRNA and of the encoded protein domains are indicated. *UTR*, untranslated region.

RESULTS

Detection of the AIB1-Δ3 Isoform—In this study we determined whether there are naturally occurring splice variants of

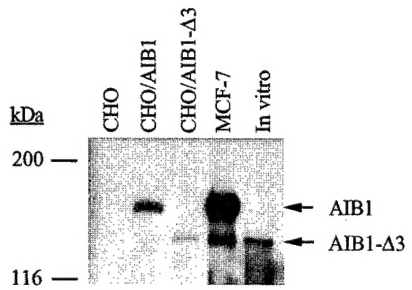

FIG. 2. **Immunoblot analysis of AIB1 isoforms in extracts of MCF-7 cells and transfected CHO cells.** Extracts of untransfected CHO cells or CHO cells transfected with plasmids encoding AIB1 or AIB1-Δ3, or MCF-7 cells were fractionated by electrophoresis on high resolution 4% polyacrylamide gels containing Tris-glycine, and the separated proteins were transferred to a nitrocellulose membrane and probed with a monoclonal antibody specific for amino acids 376–389 of human AIB1. The products of *in vitro* transcription-translation of *AIB1-Δ3* cDNA were similarly analyzed.

AIB1 present in breast cancer cells that might encode proteins with altered function relevant to breast cancer progression. The exon-intron structure of *AIB1* was assembled as shown in Fig. 1*a* by comparing the published sequence of the cDNA (14) with the contiguous genomic sequence available through the NCBI data base. We arbitrarily designated the most 5′ exon of *AIB1* as exon 1, with the result that the first codon is located in exon 2. Our initial strategy was to determine whether RNA from MCF-7 cells, which overexpress AIB1 (14), contained any splice variant forms of AIB1 RNA. To achieve this end, we performed reverse transcription and polymerase chain reaction (RT-PCR) analysis of total RNA from MCF-7 human breast cancer cells with primers amplifying the region between exons 1 and 9. This revealed two PCR products that differed in size by ~150 base pairs. These PCR products were then subjected to Southern blot analysis, and individual lanes from the membrane were probed separately with oligonucleotides specific for each exon from 2 to 8. Typical hybridizations with exons 2, 3, and 8 are shown in Fig. 1*b*. This analysis revealed that the smaller PCR product hybridized with all probes except the one specific for exon 3 (Fig. 1*b*), indicating that the lower band corresponds to an RNA splice variant (designated *AIB1-Δ3*) of *AIB1* that lacks the exon 3 sequence. We subsequently subcloned and sequenced this PCR product, confirming that nucleotides 267–439 (exon 3) of the full-length *AIB1* cDNA were missing (Fig. 1*c*).

Translation of the AIB1-Δ3 mRNA in Vitro and in Vivo—To determine whether an AIB1-related protein was encoded by the *AIB1-Δ3* mRNA, we performed *in vitro* transcription and translation of *AIB1-Δ3* cDNA. Western blot analysis with an AIB1-specific antibody of the proteins translated *in vitro* revealed the production of a 130-kDa protein (Fig. 2). Interestingly, we had also consistently detected a similar 130-kDa protein, in addition to the 156-kDa full-length AIB1, by immunoblot analysis of MCF-7 cell extracts with antibodies to AIB1 on 5–20% polyacrylamide gels (27). To determine whether the MCF-7 130-kDa species and the *in vitro* transcription translation product had identical electrophoretic properties, we performed high resolution electrophoresis on 4% polyacrylamide gels containing Tris-glycine followed by immunoblot analysis. This analysis demonstrated that the mobility of the 130-kDa protein detected in MCF-7 cell extracts was identical to that of the 130-kDa protein produced by *in vitro* transcription and translation of *AIB1-Δ3* cDNA (Fig. 2). This observation suggested that the 130-kDa MCF-7 cell protein was translated from *AIB1-Δ3* mRNA present in these cells.

To verify that the *AIB1-Δ3* mRNA was translated *in vivo* we performed transient transfection of CHO cells (Fig. 2; see Fig. 4*a*) or COS-1 cells (see Fig. 5*a*) with the *AIB1-Δ3* cDNA. Analysis of cell extracts demonstrated that this indeed resulted in the production of a 130-kDa protein, whereas transfection with the full-length *AIB1* cDNA yielded only the 156-kDa full-length protein. This latter observation demonstrated that the 130-kDa protein was clearly not the product of proteolytic processing of the full-length protein. The electrophoretic mobility of the 130-kDa protein synthesized in cells transfected with the *AIB1-Δ3* cDNA was identical to that of both the 130-kDa AIB1 species present in MCF-7 cell extracts and the product of *in vitro* transcription-translation of the *AIB1-Δ3* cDNA (Fig. 2). Together these data indicated that the endogenous *AIB1-Δ3* mRNA present in MCF-7 cells encodes a 130-kDa protein.

An examination of the sequence of *AIB1-Δ3* mRNA indicated that the open reading frame of *AIB1*, which initiates at nucleotide 184 in the full-length mRNA would terminate after 90 amino acids in the splice variant (Fig. 1*c*). We did not detect this predicted low molecular mass product *in vivo* or *in vitro*. The 130-kDa species is detected by an AIB1 antibody raised against amino acids 376–389 in the amino terminus of the protein. This suggests that the AIB1-Δ3 isoform most likely represents an NH_2-terminally truncated form of AIB1, with synthesis being initiated at an internal translation start site downstream of the splice junction but prior to amino acid 376. Such internal translational initiation has been described for various mRNAs with extended 5′-untranslated regions (34–37). The difference in size between the 156-kDa full-length AIB1 protein and the 130-kDa species suggested that the latter lacks ~210 amino acids of the former, including all of the bHLH region (residues 16–88) and most of the PAS A domain (residues 116–171) (Fig. 1*c*). This would place the initiation codon for the 130-kDa protein most likely at the codon at position 778 (Fig. 1*c*). Interestingly, for cells transfected with equivalent amounts of cDNA, the intracellular concentration of AIB1-Δ3 protein was ~10% of that of full-length AIB1 (Fig. 2; see Figs. 4*a* and 5*a*), suggesting that translation initiation of the splice variant was inefficient, possibly because of the long 5′-untranslated region of the *AIB1-Δ3* mRNA.

AIB1-Δ3 mRNA Is Overexpressed in Human Breast Cancer—Given that we first detected the *AIB1-Δ3* splice variant in a breast cancer cell line, we next examined whether its expression was restricted to tumor cells. MCF-7 cells are derived from a pleural effusion of metastatic breast cancer, whereas MCF-10A and A1N4 cells are not malignantly transformed and were derived from atypical human breast epithelial hyperplasia (38) and from human mammary epithelial cells treated with benzopyrene (39), respectively. RT-PCR followed by Southern blot analysis revealed that the amounts of *AIB1-Δ3* mRNA in MCF-10A and A1N4 cells were lower than that of MCF-7 cells (Fig. 3*a*). By subsequent real-time PCR analysis, using primers specific for AIB1 or its isoform, we have assessed that the ratio of *AIB1-Δ3* mRNA/full-length AIB1 is 5% in MCF-7 cells, whereas in MCF-10A and AIN4 cells the ratio is 0.5% (data not shown). We then compared the abundance of the *AIB1-Δ3* mRNA in a series of eight human breast tumors with that in normal tissue obtained from six women undergoing breast reduction mammoplasty. The amount of the full-length *AIB1* mRNA in tumor samples was slightly greater than that in the normal tissue samples, but this difference was not significant (Fig. 3*b*). In contrast, the abundance of the *AIB1-Δ3* mRNA in the tumor specimens was significantly greater than that in the normal tissue samples, with all but one of the tumors showing an increased amount of this transcript compared with the normal range.

Effect of the AIB1-Δ3 Isoform on Nuclear Receptor Function—We next examined the effect of the deletion of the bHLH

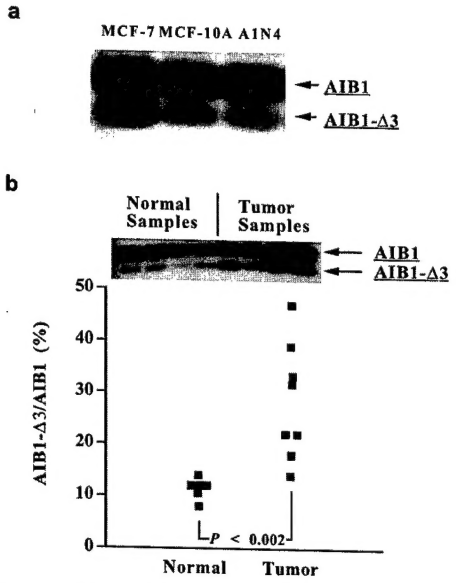

FIG. 3. **Comparison of the abundance of *AIB1-Δ3* mRNA in malignant and nonmalignant human breast tissue and cell lines.** *a*, total RNA isolated from MCF-7, MCF-10A, and A1N4 cells was subjected to RT-PCR with primers specific for exons 2 and 5 of *AIB1*. The reaction products were resolved on a 1% agarose gel and then subjected to Southern blot analysis with a ^{32}P-labeled oligonucleotide probe specific for exon 4 of *AIB1*. *b*, total RNA isolated from six normal breast and eight breast cancer tissue samples was analyzed as in *panel a*. The amounts of PCR products corresponding to *AIB1* and *AIB1-Δ3* mRNAs were quantitated by densitometry, and the abundance of the latter was expressed as a percentage of that of the former. The signal of the full-length *AIB1* transcript was compared between breast tumors and normal breast tissue with the use of an arbitrary scale; the signals in tumor and normal samples were 1.0 ± 0.46 and 0.7 ± 0.24 (means ± S.E.), respectively, and they did not differ significantly ($p > 0.05$; Student's *t* test). The *inset* shows a typical blot of 8 of the 14 samples.

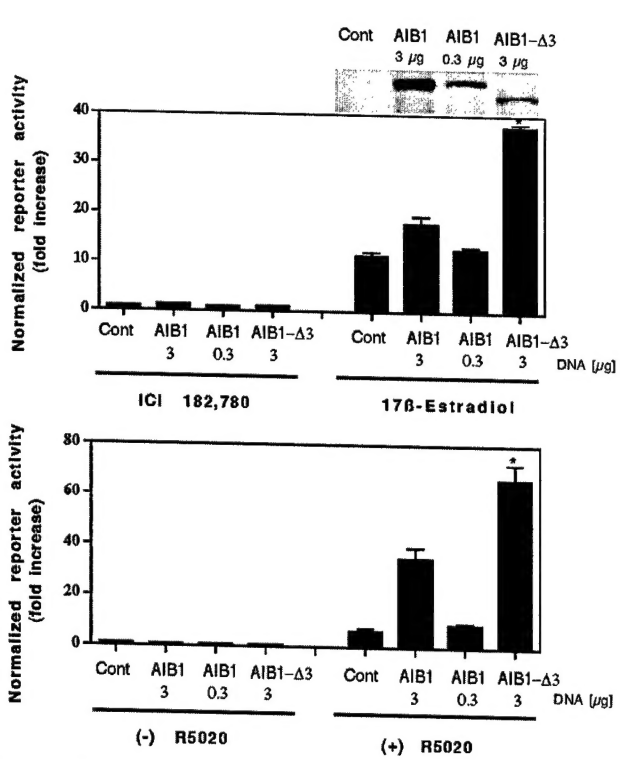

FIG. 4. **Effects of AIB1 and AIB1-Δ3 on the activation of estrogen receptor α and progesterone receptor B in CHO cells.** *a*, cells were transfected with either the empty pcDNA3 vector (3 μg) (*Cont*), pcDNA3-AIB1 (0.3 or 3 μg), or pcDNA3-AIB1-Δ3 (3 μg) together with an expression vector for human estrogen receptor α (100 ng), an ERE-luciferase reporter plasmid (1 μg), and pRL-CMV (0.1 ng). After incubation for 24 h with either 10 nM 17β-estradiol or 100 nM estrogen receptor antagonist ICI 182,780, cells were lysed and assayed for luciferase activity. The *inset* shows immunoblot analysis of transfected cell lysates that were fractionated on 4–20% polyacrylamide gradient gels and probed with antibodies to AIB1. *b*, cells were transfected as in *panel a* with the exception that the estrogen receptor vector was replaced with a vector for human progesterone receptor B (20 ng) and the ERE-luciferase plasmid was replaced by a luciferase reporter construct containing the mouse mammary tumor virus promoter (2 μg). Cells were incubated for 24 h in the absence or presence of the progesterone analog R5020 (1 nM) before preparation of lysates for luciferase assay. The firefly luciferase activity of cell lysates was divided by the *Renilla* luciferase activity (internal control), and this ratio (normalized reporter activity) for control cells incubated in the absence of agonist was assigned a value of 1. Data are means ± S.E. of values from three independent experiments, each performed in triplicate. *, $p < 0.005$ *versus* corresponding value for cells transfected with 3 μg of the AIB1 vector (Student's *t* test).

and PAS A domains in AIB1-Δ3 on protein function. AIB1 acts as a coactivator for several nuclear receptors, including those for estrogen and progesterone, which are important in breast carcinogenesis. We therefore transfected CHO cells with expression vectors encoding full-length AIB1 or AIB1-Δ3, an expression vector for estrogen receptor α, and a luciferase reporter plasmid containing an ERE. Transfection of CHO cells with 3 μg of the AIB1 expression vector resulted in a 1.4-fold increase in estrogen-induced luciferase activity, whereas transfection with 3 μg of the vector for AIB1-Δ3 resulted in a 3.8-fold increase in the estrogen response (Fig. 4*a*). However, given that the abundance of recombinant AIB1 in the transfected cells was about 10 times that of AIB-Δ3, we also transfected CHO cells with 0.3 μg of the AIB1 vector, which yielded about the same amount of intracellular recombinant protein as did 3 μg of the AIB1-Δ3 vector (Fig. 4*a*). A comparison of transfected cells containing approximately equal amounts of recombinant protein thus revealed that AIB1 and AIB1-Δ3 potentiated the estrogen response by factors of 1.1 and 3.8, respectively. Similar transfection experiments with COS-1 cells (which express endogenous AIB1) also demonstrated a greater potentiation of the estrogen response by AIB1-Δ3 than by full-length AIB1 (Fig. 5*a*). The differences between full-length and the AIB1-Δ3 isoform were seen at different concentrations of estrogen (0.1–10 nM) and thus were not due to a change in the affinity of the hormone for its receptor but rather suggest enhanced efficacy of the signaling (data not shown). We also obtained similar results in COS-1 cells with an expression vector encoding progesterone receptor B; the transcriptional response to the progesterone analog R5020 was thus potentiated to a greater extent by AIB1-Δ3 than by AIB1 in both CHO and COS-1 cells (Figs. 4*b* and 5*b*). Of particular note is that small amounts of transfected AIB-Δ3 protein had significant effects on ER- and progesterone receptor-induced transcription even against a relatively high background of full-length AIB1 (Fig. 5, *a* and *b*).

Effect of the AIB1-Δ3 Isoform on EGF Signaling—The fact that members of the p160 SRC family act as coactivators in intracellular signaling pathways that activate transcription factors other than nuclear receptors (30, 31) prompted us to examine whether AIB1-Δ3 might be able to sensitize breast cancer cells to growth factor signaling. Overexpression of members of the families of epidermal growth factor (EGF) ligands or EGF receptors is important in the malignant progression of breast cancer (40). Such growth factors also contribute to the hormone-independent phenotype of breast tumors and the HER-2 receptor is a target of current therapies (41). To determine whether AIB1 and AIB1-Δ3 affect EGF signaling, we transfected ME-180 human squamous cell carcinoma cells with the respective expression vectors and with a luciferase reporter plasmid containing the promoter of the fibroblast growth factor-binding protein (*FGF-BP*) gene. FGF-BP functions as an

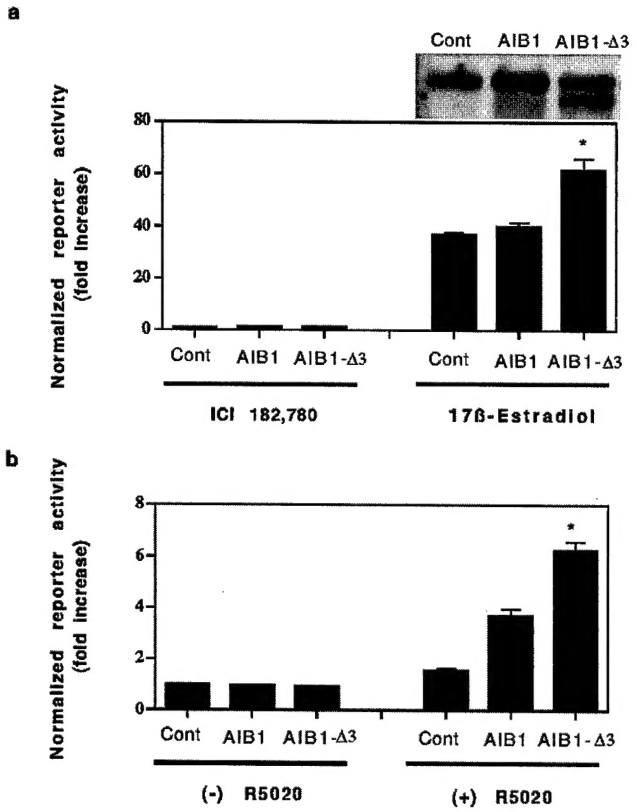

FIG. 5. **Effects of AIB1 and AIB1-Δ3 on the activation of estrogen receptor α and progesterone receptor B in COS-1 cells.** *a*, cells were transfected and analyzed as described in the legend for Fig. 4*a* (the amount of pcDNA3-AIB1 was 3 μg). *b*, cells were transfected with 1 μg of either pcDNA3, pcDNA3-AIB1, or pcDNA-AIB1-Δ3 together with an expression vector for human progesterone receptor B (10 ng), a luciferase reporter plasmid containing the mouse mammary tumor virus promoter (1 μg), and pRL-CMV (0.1 ng). After incubation for 24 h in the absence or presence of 0.5 nM R5020, cells were lysed and assayed for luciferase activity. Data are means ± S.E. of values from three independent experiments, each performed in triplicate. *, $p < 0.005$ *versus* the corresponding value for cells transfected with the AIB1 vector.

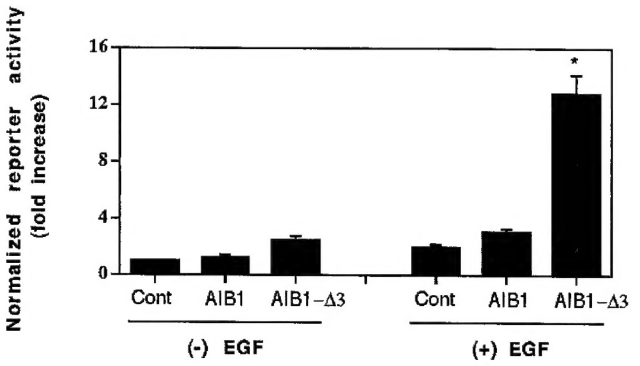

FIG. 6. **Effects of AIB1 and AIB1-Δ3 on activation of the *FGF-BP* gene promoter by EGF in ME-180 cells.** Cells were transfected with 3 μg of either pcDNA3, pcDNA3-AIB1, or pcDNA3-AIB1-Δ3 together with a luciferase reporter plasmid containing the human *FGF-BP* gene promoter (1 μg). After incubation of cells for 18 h in the absence or presence of EGF (5 ng/ml) in serum-free medium, cell extracts were prepared and assayed for luciferase activity. Activity was normalized by protein concentration, and the normalized activity values were then expressed relative to that of cells transfected with pcDNA3 and not exposed to EGF. Data are means ± S.E. of values from three independent experiments, each performed in triplicate. *, $p < 0.01$ *versus* the corresponding value for control cells.

angiogenic switch molecule (42) that is overexpressed in breast cancer, and its gene is activated by EGF in squamous cell and breast cancer cell lines (32). As reported previously, EGF induced a 2.5-fold increase in reporter activity in control cells transfected with the empty expression vector (Fig. 6). The basal EGF induction was increased slightly by transfection of the full-length AIB1 expression vector, whereas EGF induction was increased ~6-fold by expressing recombinant AIB1-Δ3.

DISCUSSION

In this study we have provided evidence for the presence of a splice variant of AIB1 that has exon 3 deleted. The *AIB1-Δ3* mRNA is translated *in vivo* in breast cancer cells into an NH_2-terminal truncated form of AIB1 that has several properties of interest. The first is that on a per molecule basis it is a more potent transcriptional coactivator of both the estrogen and progesterone receptors than the full-length AIB1 protein. This result was unexpected given that previous studies of NH_2-terminal deletion mutants of the AIB1-related protein SRC-1 did not reveal an impact of this region on nuclear receptor signaling (9, 30). One possible reason for the increased activity of AIB1-Δ3 is that the conformation of this isoform is more favorable than that of the full-length protein for interaction with nuclear receptors or for recruitment of other coactivators such as CBP/p300. An alternative possibility is suggested by the observation that the bHLH-PAS domain of SRC-1 interacts with and potentiate the activity of members of the TEF (transcription-enhancing factor) family of transcription factors (30). Thus, full-length AIB1 might be unavailable for interaction with nuclear receptors because it is sequestered or squelched by other intracellular factors. In contrast, AIB1-Δ3, which lacks an intact bHLH-PAS domain, would not bind to factors such as TEF and would be available for nuclear receptor coactivation. This notion might explain why relatively small amounts of recombinant AIB1-Δ3 are able to induce significant potentiation of nuclear receptor activity in transfected COS-1 cells with a high background of endogenous full-length AIB1. This model also predicts that the relative coactivating effects of AIB1 and AIB1-Δ3 might be cell type-specific, depending on the endogenous expression of AIB1-sequestering molecules such as TEF. Interestingly, a recent report has described that the human MMS19 protein can interact with the PAS-HLH domain of AIB1 and can regulate ER-mediated transcriptional activity (43). It is possible that the lack of interaction of AIB1-Δ3 with this protein may explain some of its increased effectiveness *in vivo*. Whatever the reason for the increased activity of the AIB1-Δ3 isoform, our data suggest that its expression would sensitize cells to the effects of estrogen and progesterone.

The second interesting aspect of the function of the AIB1-Δ3 isoform was that it also potently increased EGF signaling in ME-180 squamous carcinoma cells. This could be through direct interactions with a nuclear receptor. However, our analysis of the fragment of the *FGF-BP* gene promoter (nucleotides −118 to +62, relative to the transcription start site) used in this study did not reveal obvious consensus recognition sites for known nuclear receptors. In fact, EGF induction of this promoter is dependent on the factors AP-1 and c/EBPβ (32), either of which may interact directly or indirectly with AIB1. Alternatively it may be that a common intermediary of both nuclear receptor and AP-1 signaling such as CBP/p300 (44, 45) may be the target of the superactivating effects of the AIB1-Δ3 isoform. Whatever the mechanism of the increased potentiation of growth factor signaling by the AIB1-Δ3 isoform, our data suggest that an increase in the abundance of the AIB1-Δ3 isoform in mammary epithelial cells may be an important step in tumor progression and in the development of a more aggressive, hormone-independent phenotype.

Finally, of major interest for breast cancer is our finding that the *AIB1-Δ3* mRNA is overexpressed in breast cancer cell lines

and in human breast tumors. Our analysis of tumor cell lines suggests that there is an overall increase in the *AIB1-Δ3* mRNA relative to the full-length AIB1, although we do not know whether this is related to the gene amplification status of the endogenous gene. Alternatively, the increase in *AIB1-Δ3* mRNA may be because of an increase in RNA splicing of exon 3 in breast cancer cells. It is also possible that the increase in expression in tumors may be due in part to dilution effects of surrounding stromal tissue, but this seems unlikely given that we also see lower *AIB1-Δ3* mRNA expression in non-transformed *versus* malignant mammary epithelial cell lines. To date a number of laboratories, including ours, have reported overexpression of *AIB1* mRNA and protein in breast tumor tissue, although the assessment of the portion of breast cancers overexpressing AIB1 varies widely between groups (14, 27, 46, 47). In addition, some groups have determined that AIB1 overexpression is correlated with ER and progesterone receptor status (26), whereas others have found an inverse relationship with steroid receptor expression but a positive correlation with HER-2 and p53 expression (47). However, all of these RT-PCR or immunohistochemical analyses of expression levels have not distinguished the AIB1-Δ3 isoform signal from that of the wild type. Our data indicates that the overexpression of relatively low levels of the AIB1-Δ3 isoform can sensitize cells to estrogen, progesterone, and growth factors. Therefore we believe that measurement of increased levels of AIB1-Δ3 levels might be a sensitive indicator of the progression of breast cancer to a more hormone-independent phenotype.

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